Metformin, an antidiabetic drug, reduces neurodegeneration in rat hippocampus by disrupting adenosine receptor signalling

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ABSTRACT

Adenosine signalling has been implicated in the pathophysiology of CNS disorders, such as stroke, epilepsy and Parkinson's disease (PD). Recent reports suggest that prolonged activation of adenosine A1 and A2A receptors during stroke increases hippocampal neurodegeneration. Epidemiological studies suggested that the anti-diabetic drug metformin could promote neuroprotection in stroke patients, but the precise mechanism of this neuroprotective property remains unclear, and current literature was often conflicting. Based on pilot studies carried out initially in *ex vivo* rat hippocampal brain slices, we investigated our hypothesis that metformin binds to adenosine receptors to mediate neuroprotection. Using *in vivo* administration of adenosine A1 receptor agonist $N^{(6)}$cyclopentyladenosine (CPA), with or without the A1R antagonist dipropylcyclopentylxanthine (DPCPX), and elevation of endogenous adenosine in our *ex-vivo* hypoxia-reperfusion model, we aimed to characterize the effects of metformin co-treatments on the electrophysiological, biochemical, and morphological changes in rat hippocampus and correlated these with behavioral outcomes. Results indicated that metformin treatments prevented synaptic depression induced by CPA administration or hypoxia treatments. Moreover, metformin reduced adenosine A1 receptor-mediated hippocampal neurodegeneration and behavioral deficits. Finally, radioligand binding studies revealed a potential binding affinity of metformin to A1Rs from hippocampal membranes. In conclusion, the clinically approved metformin was effective in preventing A1R-mediated hippocampal neuronal damage, synaptic depression and accompanying behavioral abnormalities. Metformin, with partial binding affinity to at least the A1R, exhibits neuroprotective properties by acting to antagonize the effects of endogenous adenosine during ischemic conditions. This study provides support that chronic A1R stimulation promotes neurodegeneration and behavioral deficits that can be partially inhibited by the clinically approved and putative A1R antagonist metformin.

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"I can do all things through Christ who strengthens me." **Philippians 4:13**

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LIST OF ABBREVIATIONS

A1R	Adenosine A1 receptor
A2AR	Adenosine A2A receptor
A2BR	Adenosine A2B receptor
A3R	Adenosine A3 receptor
AC	Adenylate cyclase
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AGEs	Advanced glycation end products
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMPK	Adenosine monophosphate activated protein kinase
ANOVA	Analysis of variance
APSP	Adenosine induced post synaptic potentiation
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CCAC	Canadian council on animal care
CNS	Central nervous system
CPA	$N^{(6)}$cyclopentyladenosine

CPM	Counts per minute
CO_2	Carbon dioxide
ddH2O	Double distilled water
DMSO	Dimethyl sulfoxide
DPCPX	Dipropylcyclopentylxanthine
DPP	Dipeptidyl peptidase
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
fEPSP	Field excitatory post synaptic potential
GluA1	Glutamate ionotropic receptor AMPA type subunit 1
GluA2	Glutamate ionotropic receptor AMPA type subunit 2
GLUT1	Glucose transporter 1
GLUT 2	Glucose transporter 2
GLUT 3	Glucose transporter 3
GLUT 4	Glucose transporter 4
GLP	Glucagon-like peptide
GPCR	G-protein coupled receptor
HC	Hippocampus
HD	Huntington's disease
ICAM-1	Intercellular adhesion molecule 1
IHC	Immunohistochemistry
IP	Intraperitoneal
JNK	c-Jun N-terminal kinase

LKB1	Liver kinase B1
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
Mtor	Mechanistic target of rapamycin
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National institutes of health
O_2	Oxygen
OCT	Optimum cutting temperature embedding media
PBS	Phosphate buffer saline
PD	Parkinson's disease
PI	Propidium iodide
PKC	Protein kinase C
pp2A	Protein phosphatase 2A
PVD	Pial vessel disruption
PVDF	Polyvinylidene difluoride
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
r-TPA	Recombinant tissue plasminogen activator
SEM	Standard error of the mean
SGLT	Sodium-glucose co-transporter
T2D	Type II diabetes
TBST	Tris buffered saline with tween-20

TIA	Transient ischemic attacks
TNF-α	Tumor necrosis factor alpha
TZD	Thiazolidinedione
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
WHO	World health organisation

1. INTRODUCTION

1.1 Stroke

1.1.1 Stroke Pathology

In Canada, stroke is the third leading cause of death according to the Heart and Stroke Association (Jolly, Whelan, & Davy, 2018). Stroke causes cognitive problems, mood disorders, anxiety, and motor deficits (Jolly et al., 2018; Murphy & Corbett, 2009), and is the leading cause of disability in Canada (Jolly et al., 2018).

There are two different types of stroke, ischemic and hemorrhagic stroke, both having different pathophysiologies, phenotypes, symptoms, and after-effects. Ischemic stroke is defined as a loss of oxygen supply to the surrounding tissues (Hanna, 2009). A cerebral vessel becomes blocked by a clot, resulting in neuronal cells being starved of oxygen and later entering a "distressed" physiological state (e.g., oxidative and nitrosative stress and pro-inflammatory reactions) (Amantea, Nappi, Bernardi, Bagetta, & Corasaniti, 2009). This ultimately leads to increased apoptotic and overall cell death in the surrounding brain areas (George & Steinberg, 2015). These tissues are deprived not only of oxygen but also of blood supply containing many macro- and micronutrients crucial for their overall cellular functioning and homeostasis activities.

Hemorrhagic stroke following vessel rupture is similar in effect but results from a loss of controlled reperfusion within the brain. This leads to an increase in intracranial pressure causing damage to the adjacent and surrounding tissues (Lefkovits et al., 1992). Hypertension, high cholesterol levels, smoking, diabetes, as well as clotting factor disorders and pathologies are risk factors for the incidence of hemorrhagic stroke (Grysiewicz, Thomas, & Pandey, 2008). These risk factors promote the vasculature wall break down and collagen replacement, which results in a decrease in compliance of the vessels increasing their susceptibility to rupture (Testai & Aiyagari, 2008).

Ischemic stroke, on the other hand, most commonly takes place in branching small to intermediate cerebral vessels, where the pressure and velocity of blood flow increases as the

size of the vessel decreases. Clots which form in the larger vessels of the vasculature, later detach and subsequently become lodged in the smaller vessels, causing a stroke to occur. 'Mini-strokes' termed as transient ischemic attacks (TIAs) happen frequently in individuals with one or more of the following conditions: hypertension, type II diabetes, cardiovascular disease, have poor diet, obese or are smokers (Grysiewicz et al., 2008). So, while these TIAs happen very rapidly, and often resolve themselves in under an hour (Albers et al., 2002) with cognitive deficits lasting 24 hours or less (Grysiewicz et al., 2008) and the sufferer noticing no significant changes or disruptions in cognition, TIAs are important predictors of stroke (Johnston, Gress, Browner, & Sidney, 2000). Studies suggest that 8-10% of TIA sufferers go on to develop stroke within 90 days of TIA with half of these individuals having strokes within the first 7 days (Rothwell & Warlow, 2005; Wolf et al., 1992), and almost 30% within 5 years (Wolf et al., 1992).

1.1.2 Stroke Interventions

Ischemic stroke therapy consists of the immediate administration of recombinant tissue plasminogen activator (r-tPA) or aspirin within the first 3 to 4.5 hours of stroke onset to resolve any blockages depriving the tissues of oxygen (Hacke et al., 2008). This helps to limit the time the tissues were kept under anoxic conditions (George & Steinberg, 2015). However, it was also noted that fewer than 2% of stroke patients receive this treatment as a result of delays in stroke diagnoses and subsequent late admissions to stroke centres (Hacke et al., 2008). R-tPA treatment also does not address the secondary concern of increased immune cell infiltration to the infarct zone and penumbra following stroke (Amantea et al., 2009; Walz & Cayabyab, 2017).

Hemorrhagic stroke therapy consists of surgical intervention for major bleeds or the administration of IV fluids and medications for minor bleeds (Dorsch, 1997). Hemorrhagic stroke only makes up 10% of all stroke cases while ischemic stroke makes up 90% of all stroke cases (Grysiewicz et al., 2008).

Therefore, most preclinical stroke research focuses on understanding ischemic stroke pathophysiology, and intracellular post-stroke signalling mechanisms to elucidate novel therapeutic targets. This includes investigating receptors, proteins, and downstream signalling molecules that change in structure, function, and quantity during stroke.

Previous studies have identified adenosine as a neuromodulator of interest during hypoxic and ischemic conditions (Nieber, Eschke, & Brand, 1999; Rudolphi, Schubert, Parkinson, & Fredholm, 1992), but the specific role of adenosine in post-stroke pathophysiology is currently under investigation in our lab (Z. Chen et al., 2014; Stockwell, Chen, Niazi, Nosib, & Cayabyab, 2016; Stockwell, Jakova, & Cayabyab, 2017)

1.2 Adenosine

1.2.1 Adenosine Signalling

Adenosine is a ubiquitous neuromodulator which mediates and modulates cell signalling (Borea, Varani, Gessi, Merighi, & Vincenzi, 2018). It can act as an intermediate at both presynaptic and postsynaptic nerve terminals to affect synaptic transmission and synapse responses (Chu et al., 2013). However, like an autocrine factor, its effects are often transient and short lived in action (A. Briones, 2014). With a half-life of less than ten seconds (Klabunde, 1983), and differing affinities for each of its four receptor subtypes (Dunwiddie & Masino, 2001), adenosine signalling rapidly becomes both complex and varied in action.

1.2.2 Adenosine Cycling

At rest, extracellular concentrations of adenosine range between 30-300nM (Borea et al., 2018; Dunwiddie & Masino, 2001) and intracellular adenosine concentrations remain stable at approximately 50nM (Bradley, Harris, & Jenner, 2005).

However, following ischemia, hypoxia, trauma, inflammation, oxidative and nitrosative stress (Haskó, Linden, Cronstein, & Pacher, 2008), seizures, pain, diabetes, cancer (Borea et al., 2018), metabolic poisoning and field stimulation of brain slices, there is an imbalance between energy supply and energy demand. This leads to the increase of extracellular adenosine levels via different pathways and mechanisms depending on the presenting stimuli (Bradley et al., 2005).

Adenosine triphosphate (ATP) can be moved out of the cell using connexin channels and anion transport channels or via vesicular transport. This extracellular ATP can then be converted to adenosine via an ecto-nucleoside triphosphate diphosphohydrolase enzyme. Or alternatively

intracellular ATP can be converted within the cell to adenosine via 5'-nucleotidase, and then moved out of the cell via an equilibrative bidirectional nucleoside transporter (Bradley et al., 2005).

Either of these listed pathways will cause massive increases in extracellular adenosine levels, and are thought to occur at nerve terminals and also in surrounding non-neuronal cells like astrocytes and dendritic cells. However, although the equilibrative nucleoside transporters are widely believed to be major players in controlling extracellular adenosine levels both at baseline and in pathophysiological conditions, such as stroke, or seizures (Baldwin et al., 2004), concentrative nucleoside transporters are not involved in extracellular adenosine increase. These concentrative transporters are only able to move nucleosides with sodium ions into the cell from the extracellular space (Pastor-Anglada & Pérez-Torras, 2018).

At low extracellular adenosine levels, adenosine is cleared by adenosine kinase which converts adenosine to adenosine monophosphate (AMP). Inhibitors of this enzyme have been shown to cause increases in extracellular adenosine under normal physiological conditions (Lloyd & Fredholm, 1995; Sciotti & Van Wylen, 1993). At high extracellular adenosine levels, adenosine is not cleared by adenosine kinase, instead adenosine deaminase was found to play a pivotal role by converting adenosine into inosine. Inhibitors of this enzyme during times of metabolic insult showed increases in extracellular adenosine (Rochon, Rousse, & Robitaille, 2001).

As ATP levels are usually 100,000 times greater than adenosine levels we can therefore observe significant changes in adenosine levels without observing major changes in ATP levels (Bradley et al., 2005). However, during stroke there is a 100-fold post-stroke elevation of extracellular adenosine (Bertil B Fredholm, Irenius, Kull, & Schulte, 2001), and this supraphysiological adenosine level remains significantly elevated for several weeks.

1.2.3 Adenosine and its Receptors

With an increased availability of adenosine to bind to its receptors, it becomes increasingly important to look at the function of each adenosine receptor subtype and how its activation is implicated in stroke pathophysiology.

There are four known subtypes of adenosine receptor: A1, A2A, A2B and A3. These receptor subtypes are coupled to G-proteins which can be inhibitory (α_i) or excitatory (α_s) in action. An inhibitory response leads to the inhibition of adenylyl cyclase which decreases cell signalling, while an excitatory response leads to the activation of adenylyl cyclase causing an increase in cell signalling (Borea et al., 2018; B B Fredholm, IJzerman, Jacobson, Klotz, & Linden, 2001; Olah & Stiles, 1995).

Of these four subtypes of adenosine receptor, the adenosine A1 and A3 receptors are inhibitory in function, while adenosine A2A and A2B receptors are excitatory in function (Stockwell et al., 2017). Knowing that there are various distributions of adenosine receptor throughout the body, where A1 and A2A are more predominantly expressed over the A2B and A3 receptors; this tells us that adenosine-receptor binding can mediate a range of different functions depending, firstly, on the concentration of adenosine present, and secondly, on the distribution and density of adenosine receptor subtypes available.

1.2.4 Adenosine and the Brain

The hippocampus is the region of the brain involved in memory, learning, thought and higher cognitive processes (Genon, Reid, Langner, Amunts, & Eickhoff, 2018). It is implicated in stroke pathophysiology because smaller hippocampal volume is associated with brain infarcts and poorer memory (Blum et al., 2012). The understanding is that this breakdown is attributed to the unregulated transfer of molecules across the membranes (Haley & Lawrence, 2017). Additionally, a recent study in humans showed that there is an age-related breakdown of the blood brain barrier that begins in the hippocampus, and this was found to be correlated with worsening cognitive impairment (Montagne et al., 2015; Nation et al., 2019). This observation coupled with the knowledge that there are greater numbers of adenosine receptors within the hippocampus as compared to other structures of the brain (Soliman, Fathalla, & Moustafa, 2018), this can result in a wide range of adenosine-mediated responses during stress and makes the hippocampus a brain region of intense interest in stroke pathology.

1.2.5 Adenosine Increase and Ischemia

Under ischemic conditions there is increased metabolic stress as a result of oxygen and glucose deprivation to the tissues (Kim, Kim, Kwon, Park, & Chung, 2006). These conditions cause

subsequent changes in AMP/ATP ratio, with bias towards AMP and adenosine production (Beauloye et al., 2001). At rest under normal physiological conditions, basal ATP concentration is in the millimolar range with basal adenosine concentration in the nanomolar range (Chu et al., 2013). Therefore, it is not difficult to understand how small changes in ATP levels can significantly alter adenosine homeostasis (Bradley et al., 2005).

However now that we have addressed the problem of normal physiological adenosine increase and cycling and pathological adenosine increase, another important facet of adenosine signalling is its associated receptors.

1.2.6 Clinical Implications of Adenosine Increase

Adenosine is a potent agonist at adenosine A1 and A2A receptors versus adenosine A2B and A3 receptors (Bertil B Fredholm et al., 2001). This is because A1 and A2A receptors have the greatest affinity for adenosine (73nM and 150nM, respectively) and therefore sufficient tonic activation of these receptors is at work at low concentrations of adenosine, while A2B and A3 receptors have the lowest affinity for adenosine (5.1μM and 6.5μM, respectively) and therefore would only be activated when adenosine concentrations are significantly greater than they would be under normal conditions (J.-F. Chen, Lee, & Chern, 2014; Bertil B. Fredholm, 1995).

This is important because during hypoxia and ischemia in tissues with abundant adenosine A1 receptors, such as the hippocampus, it is thought that adenosine and AMP may act as retaliatory metabolites; with AMP acting intracellularly via adenosine monophosphate-activated protein kinase (AMPK) and adenosine acting extracellularly via its A1 receptors (Chu et al., 2013).

These adenosine A1 receptors (Goodman & Synder, 1982) are found on both pre-synaptic and post-synaptic sites (Deckert & Jorgensen, 1988), dendrites (Rivkees, Price, & Zhou, 1995), and axonal fibres (Swanson, Drazba, & Rivkees, 1995; D. K. Von Lubitz, 1999) within the hippocampus (Swanson et al., 1995; D. K. Von Lubitz, 1999). So whereas acute A1 receptor stimulation was initially thought to be neuroprotective (K A Jacobson, von Lubitz, Daly, & Fredholm, 1996), research has shown that chronic A1 receptor stimulation is indeed neurotoxic (Z. Chen et al., 2014; D. K. Von Lubitz, Lin, & Jacobson, 1995) with the hippocampus being one of the brain structures most significantly affected (Stockwell et al., 2017).

1.2.7 Benefits of Adenosine Receptor Activation and Inactivation

Adenosine receptor agonists and antagonists have been proposed as therapeutic agents in clinical trials in a wide array of conditions (Kenneth A Jacobson & Gao, 2006; Sachdeva & Gupta, 2013).

Therefore, we believe that by targeting some of these receptors which are known to be implicated in stroke pathology, it may be possible to provide some therapeutic benefit at the time of stroke.

1.3 Metformin

1.3.1 Type II Diabetes

Type II Diabetes (T2D) is a disease which is defined as a combination of hyperglycaemia and insulin resistance. Where normal blood glucose levels range from 4-6mmol L^{-1}, levels above this range can be an indication of the beginnings of prediabetic pathology.

1.3.2 Antidiabetics and Disease

There are 8 different classes of antidiabetic drugs, these are: biguanides, sulfonylureas, dipeptidyl peptidase (DPP) IV inhibitors, glucagon-like peptide (GLP) 1 agonists, alpha-glucosidase inhibitors, meglitinides, thiazolidinediones (TZDs) and sodium-glucose co-transporter (SGLT) 2 inhibitors. These antidiabetic drugs can be administered in combination or individually depending on an assessment of the patient's current β-cell function and the knowledge of the physician. Other factors also taken into consideration are comorbidities, the potential for drug-drug interactions, and the complexity of the treatment versus compliance. As a final line of therapy, insulin can be given in combination with these antidiabetic drugs in patients with end stage or worsening diabetic conditions which can no longer be well controlled with the current therapies.

1.3.3 Diabetes and Disease

T2D has been found to be a risk factor for the development and/or progression of neurodegeneration in epidemiological studies where data shows that the pathophysiology of T2D may overlap with PD and other age-related diseases (Hu, Jousilahti, Bidel, Antikainen, & Tuomilehto, 2007; Santiago & Potashkin, 2013).

Thus far, studies show that anti-diabetic interventions with or without insulin are neuroprotective and delay diagnoses of neurodegenerative conditions by several years (Wahlqvist et al., 2012). Preclinical studies show biguanides, TZDs and sulfonylureas have neuroprotective effects against the pathogenesis of motor-related neurodegenerative conditions like Huntington's (HD), Alzheimer's disease (AD), and Parkinson's disease (PD) in animal models (Ma et al., 2007; Martin et al., 2009; Wahlqvist et al., 2012), particularly in relation to the reduction of oxidative stress, but the specific mechanisms of action for these pathways currently remain unknown.

Additionally, hyperglycemia is a problem in T2D disease pathology because it leads to the production of advanced glycation end products (AGEs). AGEs are formed via a nonenzymatic reaction involving glucose with proteins, lipids and nucleic acids (Goh & Cooper, 2008). When AGEs bind to the receptor for AGEs (RAGE), this leads to an increase in intracellular signalling which promotes increases in oxidative stress via the reduction of nitric oxide production (Chakravarthy, Hayes, Stitt, McAuley, & Archer, 1998).; while also contributing to the increase of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), proinflammatory cytokines like TNF-α (tumour necrosis factor-α) and IL-6 (interleukin 6), as well as prosclerotic adhesion molecules such as ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) along with VEGF (vascular endothelial growth factor) (Goh & Cooper, 2008). As a result, by controlling hyperglycemia via antidiabetic medications like metformin in T2D, the potential for the overproduction of AGEs and subsequent vascular damage is greatly reduced (Marx et al., 2004; Ouslimani et al., 2007).

1.3.4 Metformin – Neuroprotection in the Brain

Metformin, a biguanide, is used as the first line of therapy for T2D, and is listed as an essential medicine by the World Health Organisation (WHO) (Knowler et al., 2002; Lu et al., 2016). It exerts its effect to increase the uptake of peripheral blood glucose levels via the activation of a molecule known as AMPK (Zhou et al., 2001). However, more recent studies now show that metformin inhibits hepatic gluconeogenesis in an AMPK/liver kinase B1 (LKB1) independent manner to decrease blood glucose levels (Foretz et al., 2010). This inhibition is thought to be related to the ability of metformin to inhibit mitochondrial glycerophosphate dehydrogenase, the redox shuttle enzyme, which plays a role in mediating cytosolic and mitochondrial redox states (Madiraju et al., 2014, 2018; Miller & Birnbaum, 2010), alluding to the idea that metformin does appear to have a significant impact on mitochondrial function.

However, outside of its antidiabetic effect, metformin has also been shown to confer therapeutic benefits for diseases such as polycystic ovary syndrome (Tang, Glanville, Orsi, Barth, & Balen, 2006), cancer (Duncan & Schmidt, 2009; Salani et al., 2014), and atherosclerosis (Mamputu, Wiernsperger, & Renier, 2003), all through different mechanisms of action.

In addition, recent preclinical studies also suggest that metformin can confer neuroprotection in the middle cerebral artery occlusion (MCAO) stroke model. Chronic metformin administration prior to stroke is thought to confer neuroprotection in the MCAO model by preventing ischemic brain injury while inducing angiogenesis (Venna, Li, Hammond, Mancini, & McCullough, 2014) and neurogenesis (Liu, Tang, Zhang, Wang, & Yang, 2014). Metformin treatment was also implicated in enhancing spatial memory formation in another study (J. Wang et al., 2012).

On the other hand, caution should be used when considering metformin as a potential novel drug for treating ischemic events. While many studies suggest that metformin has substantial neuroprotective potential, the effect of metformin in different animal models of stroke remains controversial.

In a study where metformin was administered acutely (3 days) prior to stroke, it was shown to exacerbate stroke damage and increase infarct size, while metformin administered chronically

(3 weeks) pre-stroke showed neuroprotective effects dependent on nitric oxide (Li, Benashski, Venna, & McCullough, 2010). This is thought to be in relation to the action of metformin to inhibit the production of reactive oxygen species (ROS) via its effect on mitochondrial redox processes (Lu et al., 2016; Owen, Doran, & Halestrap, 2000).

On the other hand, another study showed that the acute administration of metformin in an *in vivo* MCAO stroke model was neuroprotective. In this publication, researchers found that the activation of AMPK induced autophagy in the brain, reduced infarct volume, decreased cell apoptosis and augmented subsequent neurological deficits (T. Jiang et al., 2014). These beneficial effects were completely reversed when compound C, the AMPK inhibitor, was utilised and partially reversed when 3-methyladenine, the autophagy inhibitor, was utilised. This study highlighted the benefit of preconditioning the tissues with metformin prior to the induction of the stroke (T. Jiang et al., 2014). This was in contrast to prior findings by Li and colleagues (Li et al., 2010) where the effect of acute metformin administration during MCAO was neurotoxic and exacerbated stroke damage.

Due to the challenges in the literature in deciphering the differences in mode of administration of metformin treatment, as well as significant differences in the concentrations and doses used; the overall mechanism of metformin's neuroprotective potential in the brain has not yet been discussed in depth, and studies often seem to provide conflicting results that favour either degeneration or protection.

Additionally, within these studies there are also varying degrees of ischemic insult, various time durations and different stroke models that are being used to replicate *in vivo* strokes in patients. Therefore, while AMPK and/or metformin may prove to be neuroprotective in some studies, in others it is noted to be neurotoxic. Overall, it becomes extremely difficult to interpret these studies and to decipher the tipping point between metformin's deleterious and beneficial effects.

Another pathway of note in ischemic studies looks at the relationship between adenosine and glutamate. Both glutamate and adenosine increase during global ischemia, and studies using global ischemia models suggest that endogenous adenosine, as well as exogenous adenosine and selective A1R agonists, when acutely administered, provide neuroprotection primarily by inhibiting glutamate excitotoxicity (Dunwiddie & Masino, 2001). Given that the exogenous

administration of adenosine or selective adenosine A1 receptor agonists have different effects in stroke damage (enhancing neurotoxicity when applied chronically (D. K. Von Lubitz et al., 1994) or neuroprotection when applied acutely (K A Jacobson et al., 1996)), it is plausible to consider that the controversy surrounding metformin's effects in stroke model may be related to its potential interaction with adenosine signalling. To date, there are no studies linking the potential actions of metformin on adenosine-related proteins that may play an important role in neuroprotection or enhancing neurotoxicity. However, some studies have mentioned 5' adenosine monophosphate-activated protein kinase (AMPK) as a compound of interest in metformin-related studies.

1.3.5 5' Adenosine monophosphate-activated protein kinase (AMPK)

At this point in this thesis, it is important to highlight the role of AMPK, how it is implicated in facilitating neuroprotection, and to also answer the question of whether metformin exerts its neuroprotective effects via AMPK.

It has been found that an increase in AMP leads to the activation of AMPK which is comprised of α, β, and γ subunits (Carling, Thornton, Woods, & Sanders, 2012). The γ subunit senses the change in AMP/ATP ratio, which is an indicator of cellular energy levels and metabolic stress (Li & McCullough, 2010). Increases in AMPK activity has been noted to be responsible for improving oxidative stress (Blázquez, Woods, de Ceballos, Carling, & Guzmán, 1999), autophagy (T. Jiang et al., 2015; Manwani & McCullough, 2013; Sheng et al., 2010), apoptosis (Duan et al., 2016; T. Jiang et al., 2015), mitochondrial dysfunction (Toyama et al., 2016), glutamate excitotoxicity (Connolly, Düssmann, Anilkumar, Huber, & Prehn, 2014), neuroinflammation (Liu, Tang, Li, et al., 2014), angiogenesis (Jin et al., 2014), neurological score improvements and the amelioration of behavioral deficits (Ashabi et al., 2017; Ghadernezhad, Khalaj, Pazoki-Toroudi, Mirmasoumi, & Ashabi, 2016; Sarkaki et al., 2015).

Several neuroprotective benefits of AMPK have also been listed in the literature to be induced by the chronic administration of metformin prior to stroke (Jia, Cheng, Ni, & Zhen, 2015; Li et al., 2010). Similarly, as with metformin studies where some show neuroprotective benefits and others show neurotoxic effects, the same can be seen with AMPK-related studies.

Inhibitors of AMPK like compound C have been found to inhibit or disrupt the neuroprotective potential of AMPK during oxygen-glucose deprivation studies, which model ischemia *in vitro* (P. Wang et al., 2011). In contrast, AMPK activators like AICAR have been shown to facilitate neuroprotection in the same model (Culmsee, Monnig, Kemp, & Mattson, 2001; S. Jiang et al., 2018).

There also are some reports in the literature which suggest that metformin may exert some effects that are independent of AMPK, which therefore would directly contradict the idea that metformin is a direct activator of AMPK (Kalender et al., 2010; Rena, Pearson, & Sakamoto, 2013). This tells us that if metformin is not a direct activator of AMPK then its effects are not synonymous with AMPK studies, and it again becomes increasingly difficult to tease out metformin-dependent effects from AMPK-mediated effects. Many stroke-related studies that reported post-stroke neuroprotective effects with metformin or AMPK either did not test AMPK levels or failed to utilise AMPK inhibitors like Compound C in their experimental designs.

Overall it seems that the post-ischemic effect of metformin is dependent on three factors: duration of stroke, concentration of metformin used and duration of administration.

Given the scope of the beneficial yet controversial effects of metformin/AMPK during stroke, our study will primarily focus on understanding the effect of metformin in response to supraphysiological adenosine increases *ex-vivo* and *in vivo*.

1.4 Rationale

Firstly, we want to test whether acute or chronic A1R stimulation at the proposed concentrations mimics the adenosine elevation that occurs during stroke resulting in neuronal dysfunction and cell death. We will be using acute one-hour metformin incubations of hippocampal brain slices *ex vivo* or intraperitoneal (i.p.) injections of metformin *in vivo* thirty minutes prior to administration of adenosine A1 receptor agonist (CPA) to test whether hippocampal neurodegeneration and synaptic depression can be prevented by metformin; and whether this effect is neuroprotective and is accompanied by the attenuation of both cognitive and motor deficits in a rat animal model. We will also test the hypothesis that the anti-diabetic drug metformin interferes with adenosine A1R signalling, thereby promoting neuroprotection

by blocking CPA-induced effects. The following are brief outlines of my experimental objectives and the overall hypothesis of my project.

1.5 Hypothesis

Given that the anti-diabetic drug metformin has been implicated in modulating responses to stroke-induced brain damage, we hypothesize that metformin, when given immediately before or after a stroke, reduces neurodegeneration in rat hippocampus by disrupting adenosine A1 receptor (A1R) signalling.

1.6 Objectives

- To understand the effect of varying concentrations of metformin *in vivo* and *ex vivo* on stroke-like insults.
- To characterise the effects of metformin on hippocampal neuron health exposed to chronic A1R signalling.
- To investigate the effect of metformin on adenosine A1R signalling *in vivo* and *ex vivo*.

1.7 Clinical Relevance

With stroke being a multi-faceted disease with so many different cell types and receptors being implicated in stroke-related cell death and injury, it is important to target as many of these pathways to provide the best possible outcome for the patient. My study presents the first evidence for the potential binding activity of metformin on adenosine receptors, specifically A1Rs. Therefore, with a drug such as metformin which operates via many different mechanisms of action, we believe it could be repurposed as an inhibitor of adenosine signalling and as a secondary line of therapy to be given concurrently with r-tPA to extend the therapeutic window. Alternatively, metformin could also be administered pre-stroke to patients at high risk for the development of ischemic strokes as a preventative measure.

Given that the literature notes other mechanisms of action for metformin including increasing angiogenesis, neurogenesis, anti-inflammatory effects via microglia activation, reductions in

oxidative stress via increases in nitric oxide synthesis, anti-cancer effects via mTOR activation, increases in PP2A activity in AD, a decrease in tau phosphorylation and the reduction of huntingtin protein load in HD, it is possible that beyond stroke metformin could be beneficial for many disorders as a symptomatic treatment or as synergistic-concurrent therapy.

2. MATERIALS AND METHODS

2.1 Ethics Statement

All animal care and experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 85-23, revised 1985) and according to guidelines of the Canadian Council for Animal Care (CCAC) under the supervision of the University of Saskatchewan Committee on Animal Care and Supply (under animal protocol 20070090). All experimental designs and protocols were in accordance with ARRIVE guidelines to minimize animal suffering and the number of animals used in the study (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010; Stockwell et al., 2016).

2.2 Animal Model

Male Sprague Dawley rats purchased from Charles River, Montreal, Quebec, Canada were housed initially with their mother in cages of 16 until postnatal day 21; after day 21 they were housed in cages of 4 and later in cages of 2. Animal housing cages were changed every 2-3 days, the temperature was maintained at 20-24 degrees Celsius, with a natural 12h light-dark cycle from 7am-7pm (light) and 7pm-7am (dark). All animals had unlimited access to food pellets and water.

2.3 Live Hippocampal Slice Preparation

Male Sprague Dawley rats postnatal day 28-35 (Charles River, Canada) were anaesthetized with halothane (2-bromo-2-chloro-1,1,1-trifluoroethane Sigma-Aldrich), and decapitated. The brains were immediately removed and submerged in oxygenated, ice-cold high-sucrose dissection medium containing 87mM NaCl, 25mM $NaHCO_3$, 25mM glucose, 75mM sucrose, 2.5mM KCl, 1.25mM NaH_2PO_4, 7.0mM $MgCl_2$ and 500μM $CaCl_2$ (Brust *et al.*, 2007). Hippocampal slices were taken at 400μm thickness using a vibrating tissue slicer (VTS1200S, Vibram Instruments, Germany), and slicing was performed in the same ice-cold oxygenated dissection medium as above. Slices were maintained at room temperature for at least 1 hour in oxygenated artificial cerebrospinal fluid (aCSF) containing the following: 126mM NaCl,

2.5mM KCl, 2.0mM $MgCl_2$, 1.25mM NaH_2PO_4, 26mM $NaHCO_3$, 10mM glucose, 2.0mM $CaCl_2$ (Brust *et al.*, 2007). Oxygenation was accomplished by continually bubbling the solution with 95% O_2/5% CO_2.

2.4 Drug treatments

2.4.1 *Ex vivo* hypoxic drug incubations

Hippocampal slices were incubated with the following treatments: dimethyl sulfoxide (DMSO, vehicle control, Sigma, St. Louis, MO) and metformin hydrochloride (Metformin, Abcam) at 5µM. Incubation times for electrophysiology experiments and histology experiments varied and are specified. Metformin hydrochloride was first dissolved in double-distilled water (ddH_2O), before the same volume of DMSO used to dissolve the other drugs was added, and this solution was now added to aCSF. The final concentration of DMSO was <0.1% in each treatment.

2.4.2 *Ex vivo* acute drug incubations

Hippocampal slices were incubated with the following treatments: dimethyl sulfoxide (DMSO, vehicle control, Sigma, St. Louis, MO), N^6-cyclopentyladenosine (CPA) used as a selective adenosine A1 receptor agonist at 100nM and metformin hydrochloride (Abcam) at 5µM. Incubation times between electrophysiology experiments and histology experiments varied and are specified. All drugs with the exception of metformin hydrochloride were dissolved first in DMSO before being added to aCSF. Metformin hydrochloride was first dissolved in ddH_2O, before the same volume of DMSO used to dissolve the other drugs was added, and this solution was now added to aCSF. The final concentration of DMSO was <0.1% in each treatment.

2.4.3 *In vivo* chronic injections

Animals were injected with the following treatments: dimethyl sulfoxide (DMSO)/Saline (phosphate buffer saline 1X), N^6-cyclopentyladenosine (CPA) (3mg/kg), CPA (3mg/kg) + metformin (2mg/kg), CPA (3mg/kg) + metformin (5mg/kg) and CPA (3mg/kg) + metformin (10mg/kg). CPA was first dissolved in DMSO before PBS was added, however metformin was

dissolved in PBS first and the same volume of DMSO used to dissolve CPA was added to these treatments and our vehicle control DMSO/Saline group. Injected i.p. volumes typically ranged from 0.2-0.35ml per injection and was dependent on animal weight.

2.5 Electrophysiology

Hippocampal slices were prepared as detailed in Section **3** and submerged in an electrophysiology recording chamber with constant perfusion of oxygenated aCSF (3mL/min). Depending on the protocol used for experiments, the same concentration of drug treatment that was used for drug incubations was either added to aCSF and perfused continuously for the duration of the experiment or added directly to aCSF containing the slice for preincubation. Field excitatory postsynaptic potentials (fEPSPs) were evoked by orthodromic stimulation of the Schaffer collateral pathway using a bipolar tungsten stimulating electrode and recorded using Clampex 9.0 software (Axon Instruments, Foster City, CA). A pulled glass recording microelectrode filled with aCSF (resistance 1-3MΩ) was placed in CA1 stratum radiatum, which recorded fEPSPs induced by Schaffer collateral stimulation. fEPSPs were evoked for 0.1ms every 30s throughout recordings.

fEPSP signals were amplified 1000 times with an AC amplifier, band-pass filtered at 0.1-1000Hz, digitized at 10kHz using a Digidata 1440A digitizer (Axon Instruments) and saved to a computer as a Clampex 9.0 (Axon Instruments) file. The fEPSP response for each slice was tested using low voltage stimulation (5-6 volts) initially to test the responsiveness of the slice, high voltage stimulation was not used (9 volts+). This was to decrease the chance of random increases in population spikes during recording and to prevent extraneous damage to the axonal tracts of the slice; both of which would directly affect the level of fEPSP response recorded. Scale bar vertical calibration: 0.5 mV, horizontal calibration: 30 ms. Baseline fEPSP was set to 40-50% of the maximal fEPSP response per slice. The collected fEPSP data was analyzed using Clampfit 9.0 (Axon Instruments). fEPSP slopes were normalized to the mean of the 10 sweeps (5 min) immediately preceding drug infusion. The mean normalized fEPSP slope was plotted as a function of time with error bars representing the standard error of the mean (SEM). Sample traces are the average of 5 sweeps from a representative recording from each treatment group. All histograms show the mean normalized percent inhibition from baseline (+/- SEM). Statistical significance was assessed using one-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc analysis test where $p<0.05$=*, $p<0.01$=**, $p<0.001$=***.

2.5.1 Effect of concentration of metformin on signalling

The effect of metformin on fEPSP signalling using hippocampal slices was tested at five different concentrations (0.1nM, 1nM, 10nM, 100nM and 1μM) using electrophysiology techniques. After a stable baseline signal was achieved, each metformin concentration was added to aCSF and allowed to perfuse through the recording chamber for 20 minutes. The last 10 sweeps (5 minutes) of each treatment were averaged and compared to the baseline response. One-way ANOVA statistical testing was performed with the Tukey-Kramer post-hoc analysis test for comparison of fEPSP signalling between concentrations.

In a second set of experiments the effect of consecutive increases in metformin concentration was tested using electrophysiology techniques on hippocampal slices. After a stable baseline signal was recorded, metformin was perfused through aCSF in 15-minute intervals using 1μM, 10μM and 30μM concentrations. The average of the last 10 sweeps (5 minutes) of each treatment was averaged and compared to the baseline response. One-way ANOVA statistical testing was performed with the Tukey-Kramer post-hoc analysis test to compare fEPSP signalling between concentrations.

2.5.2 *Ex vivo* hypoxia and metformin effects

Using hippocampal slices to test the effect of hypoxia, we recorded a steady stable baseline initially with normal oxygenated aCSF before perfusing the slice for 20 minutes with a deoxygenated/hypoxic aCSF solution which was bubbled with 95% N_2, 5% CO_2. Following this the slice was again perfused with oxygenated aCSF which was continuously bubbled with 95% O_2, 5% CO_2 for approximately 1 hour and the changes to the electrophysiological fEPSP response was recorded. The average of the last 10 sweeps (5 minutes) of each segment of the experiment before the environmental conditions of the slice was changed were averaged and compared to the baseline response. One-way ANOVA statistical testing was performed with the Tukey-Kramer post-hoc analysis test to compare fEPSP signalling between baseline, hypoxia and post-hypoxia interventions.

To study the effect of metformin during hypoxic stimulation, metformin at 5μM concentration was added 5 minutes into the 20-minute hypoxia period in one experiment and in another, the slice was pre-incubated with metformin (5μM) for a minimum of 30 minutes prior to hypoxia

treatment. We chose to introduce metformin at 5 min, as this was found to be the peak period when intracellular MAPK cascades and protein phosphatases were activated (Brust *et al.*, 2006, Brust *et al.*, 2007). Once again, the average of the last 10 sweeps (5 minutes) of each treatment was averaged and statistical testing used to compare fEPSP changes with and without metformin during and after hypoxia.

2.5.3 *Ex vivo* transient adenosine increases and metformin effects

Also using hippocampal slices and electrophysiology, first, a stable baseline was attained by recording fEPSP for a minimum of 20 minutes. CPA (100nM) was then perfused through aCSF for 10 minutes and following this normal aCSF containing no drugs was perfused onto the slice during the 45-minute washout period.

In the second round of experimentation, after 1-hour preincubation with metformin (5μM), a stable baseline was recorded, following which CPA (100nM) was again perfused for 10 minutes, and a 45-minute washout period with normal aCSF followed. However, in this experiment metformin (5μM) was perfused continuously throughout the experiment to ensure the slice was continuously bathed in metformin throughout the experiment. The changes in fEPSP were compared within each experiment between baseline, CPA (100nM) treatment and the washout period as well as between experiments to compare the effect on fEPSP with and without metformin.

2.6 Histology

2.6.1 Propidium Iodide staining

Propidium Iodide (PI) is an effective fluorescent marker for indiscriminate cell death due to the fact that it only enters cells with disrupted plasma membranes. Hippocampal slices were prepared as described in Section **3** and utilized for this staining procedure. It produces strong fluorescence when excited by green light. An increase in fluorescence is indicative of increased neuronal death.

2.6.1.1 *Ex vivo* hypoxia and metformin

We wanted to examine the effect of hypoxia intervention and metformin (5μM) treatment on cell survival when metformin was given 5 minutes into hypoxia treatment as compared with metformin preincubation prior to the induction of hypoxia treatment. The methods used were adapted from Pugliese *et al*. (2009). All procedures otherwise were followed as detailed above in **6.2** with the addition of a fourth treatment group of dimethyl sulfoxide (DMSO) acting as our time control which was kept in oxygenated aCSF for the duration of the experiment.

Following hypoxia treatment, slices were treated with fresh oxygenated aCSF for 2 hours before 5μg/mL propidium iodide (Sigma) was added and incubated with the slices for 1 hour. The slices were then rinsed in aCSF and fixed in 4% paraformaldehyde in 1X PBS at 4 degrees Celsius overnight.

2.6.1.2 *Ex vivo* transient adenosine increases and metformin

We wanted to examine the effect of CPA (100nM) and metformin (5μM) treatment individually and in combination with each other on cell survival. The methods used were adapted from Pugliese *et al*. (2009) and Stockwell *et al.* (2016). All procedures otherwise were followed as detailed above in **6.3** with the exception of incubation times. The following drug treatments were added: dimethyl sulfoxide (DMSO), metformin (5μM), CPA (100nM), metformin (5μM) +CPA (100nM). Slices were incubated in DMSO, metformin (5μM) and CPA (100nM) for 1 hour, however for one treatment group metformin (5μM) was added first for 30 minutes before CPA (100nM) was added to pre-existing metformin (5μM) incubated aCSF for 1 hour. CPA treatment was administered for 1 hour at 100nM because previous experiments had revealed that CPA administration at 500nM for 30-45 minutes was sufficient to reduce the surface expression of GluA1 and GluA2 (Z. Chen et al., 2014); while also activating p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) (activated at 50nM CPA administration for 10 minutes (Brust, Cayabyab, & MacVicar, 2007; Brust, Cayabyab, Zhou, & MacVicar, 2006)) all of which are implicated in adenosine receptor mediated cytotoxic signalling pathways. aCSF was continuously kept oxygenated with 95% O_2, 5% CO_2. Following 1-hour drug incubation treatment, aCSF was replaced with fresh oxygenated aCSF and the slices were incubated for 3 hours. In the final hour of incubation 5μg/mL propidium

iodide (Sigma) was added to the aCSF. After this, slices were rinsed in aCSF and fixed in 4% paraformaldehyde in 1X PBS at 4 degrees Celsius overnight.

2.6.1.3 Propidium Iodide imaging and analysis

Slices were washed 3 x 10 minutes in 1X PBS, and mounted-on glass microscope slides (VWR) and sealed using Prolong Gold Antifade reagent (Invitrogen). After the addition of PI, all subsequent procedures were completed in the dark to prevent photobleaching.

Hippocampal slices were imaged using a Zeiss LSM700 laser scanning confocal microscope (Carl Zeiss, Germany) using a green light (543nm) to induce PI fluorescence during imaging. The whole hippocampus was imaged in pieces using a 10x objective lens, and images of CA1 pyramidal neurons were obtained using the Zeiss-Apochromat 63x/1.4 oil objective lens (Carl Zeiss). CA1 images were acquired as Z stack images of 30μm at 100μm depth into the hippocampal slice with each z stack image taken in 2μm increments. Two z stack images were taken along CA1 for each slice, with multiple slices imaged per treatment group per animal.

Data was collected with Zeiss Zen 2009 version 5.5 software (Carl Zeiss) and was analyzed using ImageJ. Z-stack images closest to the outer top and bottom of the hippocampal slice were not utilized for analysis as the neuronal damage in those areas was enhanced by the slicing procedure. Images from the inner-most 20μm (~150μm approx. deep into the slice) were combined as maximum intensity projections and were compared between treatment groups using densitometry analysis. Collected densitometry data was normalized to DMSO time control slices that were treated along with each experiment. Data was graphed as a percentage of the time control value and analyzed for significance against this control value (up to 100%) where $p<0.05$=*, $p<0.01$=**, $p<0.001$=***, $p<0.0001$=****. Full hippocampal slice images were assembled as montages of the entire hippocampus using Adobe Photoshop CS6 (Adobe Systems, Mountain View, CA). All images were taken with equal dimensions from the CA1 pyramidal cell layer, sampled at 12 bits, and were the average of two images taken side by side per slice, for a total of 4 slices per treatment group.

2.7 Biochemistry

2.7.1 *In vivo* chronic co-administration of adenosine with metformin

Animals from the same chronic injection testing group were taken, anaesthetized with halothane and decapitated. This consisted of one animal from each group: DMSO/Saline, CPA (3mg/kg), CPA (3mg/kg) + metformin (2mg/kg), CPA (3mg/kg) + metformin (5mg/kg) and CPA (3mg/kg) + metformin (10mg/kg). Following preparation, each brain was then sliced in ice-cold sucrose solution and hippocampal slices were isolated according to Section **2.3**.

Surface biotinylation, biochemistry preparation and analysis

Hippocampal brain slices after preparation were kept in aCSF that was continuously oxygenated with 95% O_2, 5% CO_2 to equilibrate for 1 hour after slicing. Following this, slices were rapidly cooled to 4 degrees Celsius with 4-degree Celsius aCSF and treated with NHS-SS-Biotin (0.5mg/mL, Thermoscientific) for 1 hour at 4 degree Celsius. The biotin reaction was quenched with quenching buffer at pH 8.3 containing 192mM glycine and 25mM Tris. Slices were then transferred to homogenization tubes containing 1% NP-40 detergent homogenization buffer at pH 8.0 which contained 50mM Tris, 150mM NaCl, 1mM EDTA, 1mM NaF, and the following protease inhibitors: 1mM PMSF, 10g/L aprotinin, 10g/mL pepstatin A, 10g/mL leupeptin, 2mM Na_3VO_4, 20mM sodium pyrophosphate, 3mM benzamidine hydrochloride and 4mM glycerol 2-phosphate.

Western blotting, imaging and analysis

Braford Assay was performed with DC Protein assay dye (Bio-Rad) to determine the protein concentration in each of the four/five treatment group lysates. Following this assay, 500µg protein lysate diluted in lysis buffer was added to Streptavidin agarose beads (Thermo scientific) and rotated overnight continuously at 4 degrees Celsius. The beads were then washed 3 times the following morning with lysis buffer containing 0.1% NP-40. A 50µl volume of 2X Laemmli sample buffer (Bio-Rad) was added and the samples were boiled for 5 minutes at 95 degrees Celsius. Samples were loaded into 10% SDS-PAGE gels and run for 15 minutes at 80V and 45 minutes at 160V or until the sample buffer has migrated to the bottom

of the glass plate. Proteins were transferred from the gel to PVDF membranes at 4 degrees Celsius on ice at 0.23A for 2.5 hours. Each membrane was then blocked in 5% blocking buffer consisting of 2.5g skimmed milk powder in Tris buffer saline with Tween-20 (TBST) for 1 hour. Then the membrane was incubated overnight in the appropriate primary antibody at 1:1000 dilution (e.g., A1R, Sigma, GAPDH, Sigma) at 4 degrees Celsius, followed by incubation of the appropriate secondary antibody for 1 hour at room temperature the next day. It was then rinsed in TBST 3 times x 10 minutes each and imaged using a Bio-Rad ChemiDoc imaging system. Following imaging, the whole cell lysate sample membrane was rinsed again 3 times x 5 minutes each and incubated for 1 hour with GAPDH (Sigma) at 1:1000 dilution, and again following this the membrane was rinsed 3 times for 10 minutes each before the secondary antibody was added also at a 1:1000 dilution for 1 hour (Sigma). The membrane was then again rinsed 3 times x 5 minutes per rinse, and the membrane was imaged with the Bio-Rad ChemiDoc imaging system. Analysis was performed using Bio-Rad Image Lab software and ImageJ (NIH). Graphs were constructed using GraphPad Prism 7 (GraphPad) with data presented as mean +/- SEM. Statistical significance was performed with a one-way ANOVA test and Tukey-Kramer post-hoc analysis test.

2.8. Behavior Experiments

Male Sprague Dawley rats post-natal day 28-35 were utilized weighing between 200-300g. Animals were handled once daily between day 28-35 for 5-7 days to familiarize the animal with the handler and all maneuvers the animal would undergo during treatment and/or behavioral testing. Animals were then injected once daily for 7 days with the following drugs: DMSO/Saline vehicle control, CPA (3mg/kg), CPA (3mg/kg) and metformin (2mg/kg), CPA (3mg/kg) and metformin (5mg/kg) or CPA (3mg/kg) and metformin (10mg/kg). On day 8 and 9 animals underwent behavioral tests of Y-Maze, Forced Swim and Open Field testing.

To begin, on the morning of testing animal cages were placed in the testing room with HEPA filters removed for 1 hour undisturbed to allow for acclimatization to room light intensities, sounds and smells within the room.

2.8.1 Y-Maze

The Y-maze was constructed using corrugated cardboard with three 45 cm x 12 cm long equidistant 120-degree arms located around a central point. Spatial cues of a colored rectangle and grey asterisk were placed in close vicinity of the maze on opposing room walls to aid with spatial awareness/perception of the animal when maneuvering throughout the maze. All other spaces concurrent to the Y-maze that would provide any confounding visual cues were covered from eyesight of the animal by a white sheet cover.

Following initial set up of the Y-Maze, one arm was blocked and sealed off to only allow for exploration of 2 arms of the maze. Each animal was placed in the start arm of the Y-Maze and allowed to explore the start and old arms without influence for 15 minutes during trial 1. Animals were then removed from the maze following the conclusion of the trial and placed back into their home cages for a minimum of 90 minutes to rest and accommodate any gained information into spatial memory. Each animal was then returned to the Y-maze with all arms open and no restrictions to access and could explore the maze freely for 5 minutes during trial 2. Both trials were recorded using a digital camera, videos were saved, and videos were scored manually and using Ethovision XT 12 software.

2.8.2 Open Field

The open field was constructed using corrugated cardboard with a defined field consisting of 16 equal squares measuring 56cm x 56cm. The centermost 4 squares were then segmented again to create an innermost square measuring 28cm x 28 cm. All other spaces concurrent to the open field that would provide any confounding visual cues were covered from eyesight of the animal by a white sheet cover. Each animal was placed in the innermost center square of the field and allowed to explore the full field without restriction for 10 minutes during the trial. Following this the animal was then removed from the field and returned to its home cage. The trial was recorded using a digital camera, videos were saved and scored manually and using Ethovision XT 12 software.

2.8.3 Forced Swim

The forced swim tank was constructed using plexiglass which had a dimension of 30 cm x 30 cm with walls measuring 60 cm high. The tank was filled with water at a temperature of 23-25 degrees Celsius to a height appropriate for the animal to be tested. At minimum the height of the water is equal to the length of the animal from the tip of its nose to the tip of its tail. Animals were placed in the tank for 10 minutes continuously throughout the trial. Following the trial, the animal was removed from the tank, dried using towels and returned to their home cage to rest. The trial was recorded using a digital camera, these videos were saved and scored manually.

2.9 Radioligand Binding Assay

After hippocampal slice preparation as detailed in Section **3**, slices were rapidly frozen in liquid nitrogen and later immersed in homogenization buffer (50mM Tris-HCl, NaCl 100mM, 1mM Ethylenediaminetetraacetic acid (EDTA), double distilled water (ddH_2O)) and homogenized using a homogenizer and dunce motions. This solution was then transferred to an ultracentrifuge tube and spun down at 26500rpm for 30 minutes at 4 degrees Celsius. The supernatant was removed and again the pellet was resuspended in binding buffer (75mM Tris-HCl, 12.5mM $MgCl_2$, 1mM EDTA, 1mg/mL bovine serum albumin (BSA), and ddH_2O) and homogenized, before it was spun a second time in an ultracentrifuge tube at 26500rpm for 30 minutes at 4 degrees Celsius. The supernatant was removed, and the pellet was resuspended in binding buffer. A Bradford assay was run to ascertain protein concentration per rat hippocampal sample and the volume needed to use 125μg/mL of protein for the assay was calculated.

DPCPX is a selective adenosine A1 receptor antagonist. We used a tritiated form of this compound, sourced from Perkin-Elmer for our radiolabeling binding assay. We compared the binding profile of H^3-DPCPX to A1R using hippocampal lysates when we increased concentrations of cold-DPCPX or cold-metformin. H^3-DPCPX was diluted to a 1:100 concentration in binding buffer. Six concentrations of cold/non-radioactive DPCPX and metformin were used to compare the binding profiles of DPCPX and metformin individually to the adenosine A1 receptor. These were: 0.1nM, 1nM, 10nM, 100nM, 1μM and 10μM. Each sample was mixed with the appropriate volume of H^3-DPCPX and cold-DPCPX or H^3-DPCPX

and cold-metformin and incubated for 2 hours at room temperature. There were 2 negative controls in this experiment, our first control was a vial containing only binding buffer and no drugs, and our second control was a vial containing binding buffer and H^3-DPCPX. The samples were then filtered in a drum using vacuum filtration, rinsed with 1X PBS and incubated in scintillation vials overnight. The following day these scintillation vials were placed in a scintillation counter and radioactive counts in counts per minute (cpm) were read and recorded from each sample. All values were normalized according to the control value which was a measure of background radiation.

Data Analysis

Each sample was run multiple times with both cold-DPCPX and cold-metformin in comparison to H^3-DPCPX, using a total of 8 individual samples of the same age from the same batch of animals. All values after normalization were averaged and plotted as a linear regression in GraphPad Prism 7 (GraphPad).

3. RESULTS

3.1 The effect of various concentrations of metformin on fEPSP signalling

To investigate the effect of metformin on adenosine signalling, we looked at the fEPSP response when different concentrations of metformin were administered. Hippocampal CA1 neurons when stimulated in the presence of 0.1nM-30μM of metformin showed an increase in field excitatory postsynaptic potential (fEPSP) (**Figure 3.1 C**). When the last 10 traces (5 minutes) of each experiment was averaged and analysed using one-way ANOVA statistical testing, significant fEPSP increases were noted with metformin treatment at 1μM and 10μM. However, rapid decreases in fEPSP signalling were observed at the 30μM concentration, resulting in the loss of statistical significance with this dose.

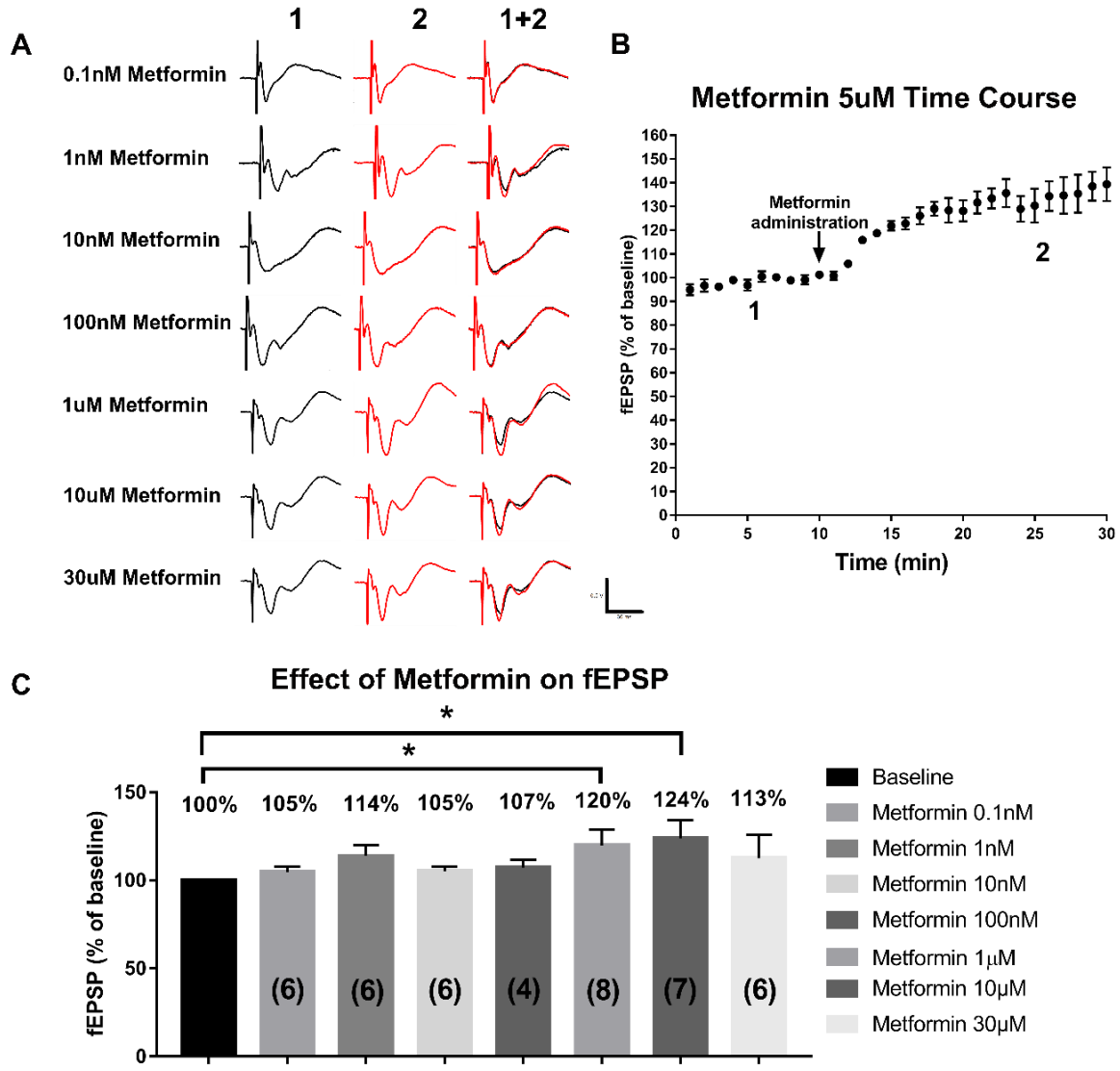

Figure 3.1: Metformin treatment significantly increased fEPSP signalling when perfused at the 1µM and 10µM concentrations. N=6 recordings from individual slices from different animals for metformin 0.1nM, 1nM and 10nM. N=4 from individual slices from different animals for metformin 100nM. N=8 recordings from individual slices from different animals for metformin 1µM, 10µM and 30µM consecutive responses. Data are mean ± SEM. Vertical calibration: 0.5 mV. Horizontal calibration: 30 ms.

Five different concentrations of metformin (0.1nM, 1nM, 10nM, 100nM, 1µM) were added to aCSF and perfused on to fresh hippocampal slices for 20 minutes, and the changes in fEPSP signalling were observed and analysed. In a second experiment, three different concentrations of metformin (1µM, 10µM and 30µM) were added to aCSF and perfused on to fresh

hippocampal slices for 15 minutes consecutively, and the changes in fEPSP signalling were observed and analysed using one-way ANOVA statistical testing. Sample traces of fEPSP responses are shown in **Figure 3.1 A**, however this data is summarised in **Figure 3.1 C**. A significant increase from baseline fEPSP signalling was observed at 1μM and 10μM concentrations.

From this study we were able to deduce that metformin treatment produces a significant effect between 1-10μM when perfused on to the slice through aCSF for 15-20 minutes. A dose response curve (not shown) gave an EC_{50} value of 4.89μM, therefore we decided for the remainder of our drug studies to use a 5μM concentration of metformin. **Figure 3.1 B** shows the effect of metformin (5μM) on fEPSP after 20 minutes of administration, which caused an increase in fEPSP. Additionally, we had observed in this experiment that metformin (5μM) was able to cause a persistent and sustained increase in fEPSP for an additional 20 minutes with constant metformin 5μM perfusion (not shown).

3.2 The effect of metformin during hypoxia treatment

Pre-clinical studies revealed that metformin administration in stroke animal models resulted in neuroprotective benefits but did not specify a mechanism of action beyond activation of AMPK (Li et al., 2010), neurogenesis and angiogenesis induction (Liu, Tang, Zhang, et al., 2014) (Abdelsaid et al., 2015) and increased microglial response (Jin et al., 2014). We utilised electrophysiology and histology techniques with hypoxia treatments and different metformin intervention times to characterise and understand the effect of metformin during hypoxia.

3.2.1 Effect of hypoxia on fEPSP with different metformin intervention time points

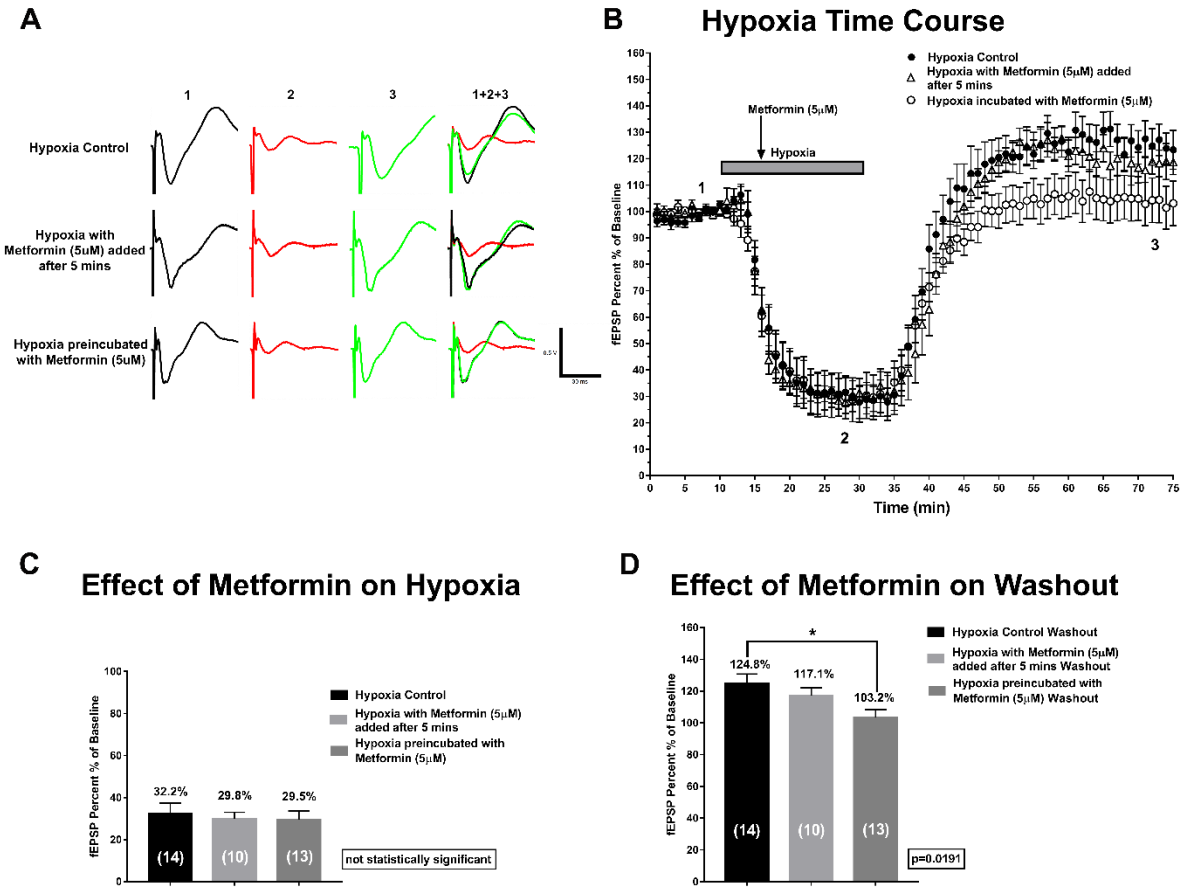

Figure 3.2.1: Metformin (5μM) had no effect on hypoxia-induced synaptic depression but preincubation with metformin at 5μM prevented hypoxia-induced adenosine-mediated postsynaptic potentiation. Recordings were from individual slices from different animals with n=14 for hypoxia treatment control, n=10 for metformin (5μM) treatment administered 5 minutes into hypoxia and n=13 for metformin (5μM) preincubation and hypoxia treatment. Sample traces for each experiment are shown in **Figure 3.2.1 A**. Data are mean ± SEM. Vertical calibration: 0.5 mV. Horizontal calibration: 30 ms.

A full comparison of each experiment showing fEPSP response prior to, during and after 20-minute hypoxia treatment (95% N_2, 5% CO_2) without metformin (5μM), with metformin (5μM) added 5 minutes into hypoxia treatment and with the preincubation of metformin (5μM) for a minimum of 30 minutes prior to the start of the experiment is shown in **Figure 3.2.1 B**.

While metformin was found in the literature to be neuroprotective against stroke in the MCAO model (Jin et al., 2014; Li et al., 2010; Liu, Tang, Zhang, et al., 2014), we found that our metformin-hypoxia experiments gave rise to some interesting results. In comparing the last 10 fEPSP traces (5 minutes) of hypoxia treatment, without metformin, when metformin (5μM) was added 5 minutes into hypoxia or with slices that were preincubated with metformin (5μM), we found that metformin treatment resulted in the same level of fEPSP in comparison to the control hypoxia treatment (**Figure 3.2.1 C**). There was no statistically significant difference in fEPSP signal at the end of hypoxia between all three treatments ($p=0.9$). It is therefore notable that metformin (5μM) treatment did not affect synaptic depression during hypoxia (**Figure 3.2.1 C**), as this is normally attributable to A1R stimulation and A1R-mediated neurotoxicity (Z. Chen et al., 2014)

Now when analysing the effect of metformin (5μM) during normoxia, we compared the last 10 fEPSP traces (5 minutes) of normoxia treatment between all three experiments (**Figure 3.2.1 D**). We noted that there was an increase in fEPSP that was observed in the control experiment which was also present when metformin (5μM) was added 5 minutes into hypoxia, and this increase was found to be statistically significant (**Figure 3.2.1 D**, where $p<0.05$=*). This increase in fEPSP after hypoxia treatment was first observed by Hsu and colleagues (Hsu & Huang, 1997), and has been since termed adenosine-induced postsynaptic potentiation (APSP) by Qin and colleagues (Qin et al., 2019, submitted). Moreover, our results showed that there was no significant difference in fEPSP signalling at the end of normoxia washout treatment between the control and metformin (5μM) response when it was added 5 minutes into hypoxia treatment. On the other hand, when metformin (5μM) was added to slices for a minimum of 30 minutes prior to the start of the hypoxia experiment and also perfused continuously throughout, our results differed.

Interestingly the development of post-hypoxic APSP was affected when slices were preincubated with metformin (5μM) prior to the induction of hypoxia. Signalling transmission returned to baseline and remained constant for 30 minutes until the end of the experiment, and there was no statistically significant difference between the fEPSP signal at the baseline as compared to the fEPSP signal at the end of normoxia (**Figure 3.2.1 D**, where $p=0.82$).

Our results overall showed that the preincubation of hippocampal slices with metformin (5μM) prevented the development of adenosine-induced post-synaptic potentiation (APSP) during normoxic washout but the addition of metformin (5μM) during hypoxia did not.

3.2.2 Effect of hypoxia on indiscriminate cell death with different metformin intervention time points

Given the interesting finding from our electrophysiology results (**Figure 3.2.1**), we decided to mirror this experiment and use propidium iodide staining and confocal imaging to determine the levels of indiscriminate cell death. We thought this would allow us to understand the effect of metformin on hypoxia treatment, and if time of administration played a crucial role in the recovery of cells as our electrophysiology experiments (**Figure 3.2.1**), and previous citations in the literature had led us to believe (Li et al., 2010).

In this additional experiment we pre-incubated slices with metformin at 5μM for one hour before adding the hypoxic aCSF solution. To other slices we incubated with DMSO for the same length of time as a control. And in the last two treatment groups we induced hypoxia in one and added metformin (5μM) after 5 minutes of hypoxia in the other. We then added propidium iodide and observed the levels of fluorescence using a confocal microscope, where increases in fluorescence are directly proportional to increases in cell death (**Figure 3.2.2 A** and **B**). We quantified this fluorescence using densitometry, and analysed our results using statistical one-way ANOVA testing in **Figure 3.2.2 C** where $p<0.05$=*, $p<0.01$=**, $p<0.001$=***, $p<0.0001$=****. N=4 individual slices per treatment group.

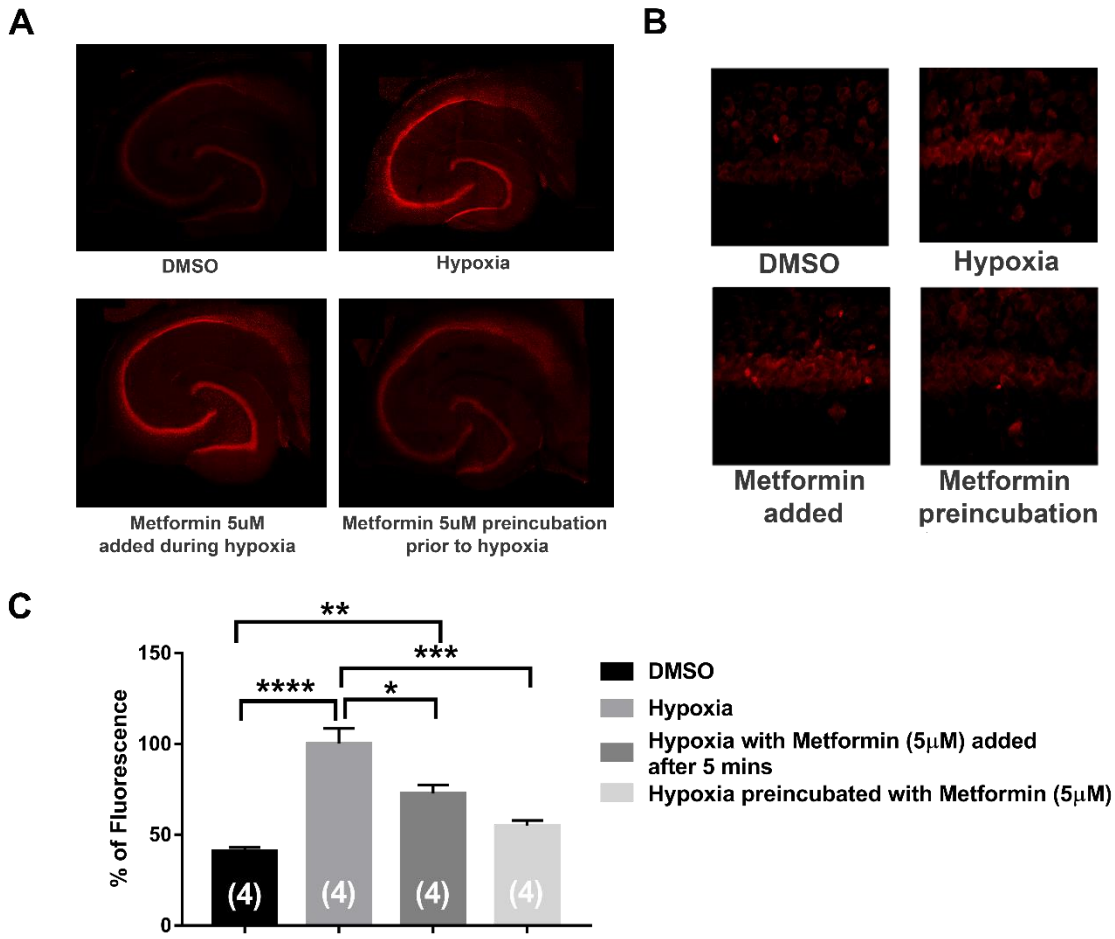

Figure 3.2.2: Metformin (5μM) preincubation prevented hypoxia-mediated cell death. N=4 individual slices per treatment group.

Hippocampal slices were pre-incubated with metformin (5μM) or DMSO for 1 hour. DMSO-treated slices remained immersed in oxygenated aCSF while all other treatment group slices were immersed in hypoxic aCSF for 20 minutes. One treatment group received metformin at 5μM 5 minutes into the 20-minute hypoxic treatment. Following this all solutions were replaced with normoxic aCSF for 45 minutes with or without metformin (5μM) to mirror our earlier electrophysiology experiments. Propidium iodide staining was used to quantify the overall cell death levels by measuring fluorescence intensities using confocal microscopy.

With confocal microscopy, a significant decrease in propidium iodide red fluorescence was observed with metformin (5μM) preincubation ($p=0.0003$) as compared to when metformin (5μM) was added 5 minutes to hypoxia treatment ($p=0.0135$) or not at all (**Figure 3.2.2 C**). It

is important to note that while metformin (5μM) treatment given 5 minutes into hypoxia treatment did significantly decrease the level of fluorescence as compared to our hypoxia positive time control result, metformin preincubation still yielded greater neuroprotection and resulted in lower levels of cell death in comparison.

3.3. The effect of metformin during transient endogenous adenosine increases

Our hypoxia study with different metformin intervention time points yielded interesting results where we found slices preincubated with metformin at 5μM showed significant improvements in neuronal cell health. Therefore given that it has previously been shown that both hypoxia and CPA treatment cause neuronal death due to activation of A1Rs (Stockwell et al., 2016), we decided to test the effect of metformin preincubation on CPA-induced A1R activation.

3.3.1 Effect of metformin on fEPSP during adenosine release

First, we looked at the effect of metformin (5μM) with CPA at 100nM on the field excitatory postsynaptic potential (fEPSP). We wanted to determine if metformin would prevent CPA-induced synaptic depression similar to effects observed with the A1R antagonist DPCPX in the presence of adenosine (Brust et al., 2006). DPCPX was also shown to cause statistically significant increases in fEPSP above baseline when perfused (Brust et al., 2006; Pascual et al., 2005).

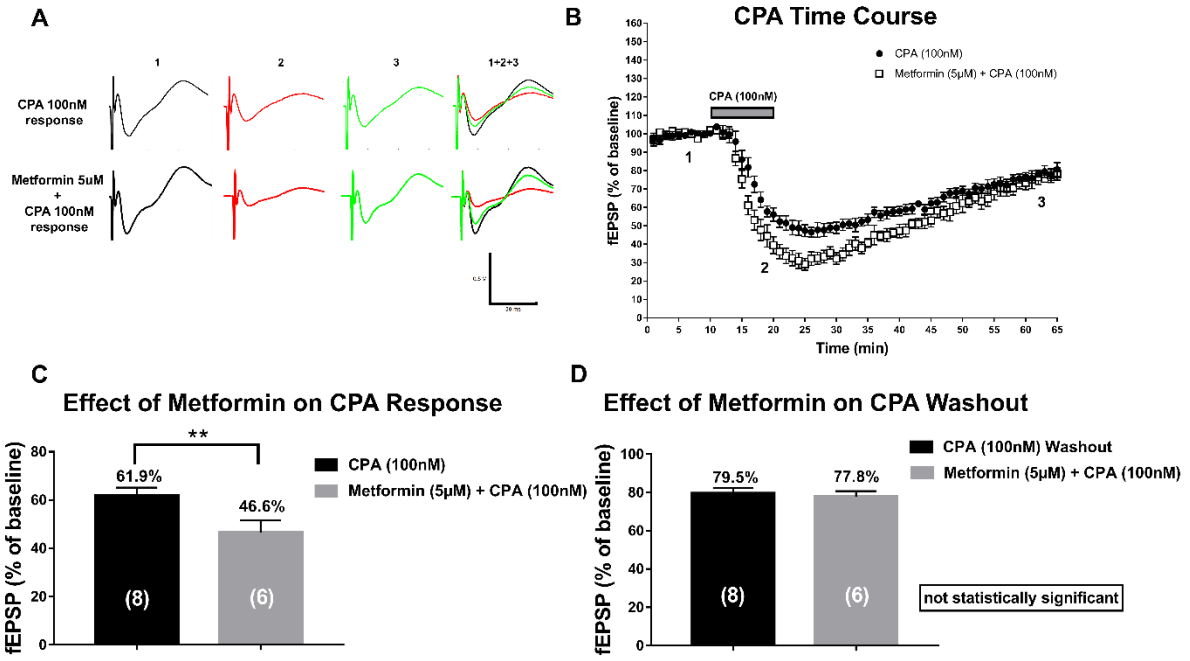

Figure 3.3.1: Preincubation with Metformin (5μM) treatment did not prevent CPA-induced decreases in fEPSP signalling. Recordings were from individual slices from different animals with N=8 for CPA (100nM) treatment and N=6 for metformin (5μM)+CPA (100nM) treatment. Data are mean ± SEM. Vertical calibration: 0.5 mV. Horizontal calibration: 30 ms.

Hippocampal slice fEPSP signals were recorded when CPA at 100nM was added to aCSF solution and perfused for 10 minutes at a rate of 3mL/min. This was then compared to hippocampal slices pre-incubated with metformin (5μM) for 30 minutes – 1 hour prior to the start of electrophysiology experiments. Baseline fEPSP signals were recorded and the slice was then perfused with aCSF containing 100 nM of CPA also in the presence of metformin (5μM). If the slice had undergone metformin preincubation, then metformin (5μM) was perfused throughout the experiment.

In comparing the fEPSP response to CPA (100nM) with or without metformin (5μM) by averaging the last 10 traces and using one-way ANOVA statistical testing, we found that hippocampal CA1 neurons when stimulated in the presence of CPA at 100nM showed a 38.1% reduction in fEPSP. This is a repetition of previous experiments conducted in our lab using CPA at 500nM (Stockwell et al., 2016). Interestingly, metformin at 5μM in the presence of

CPA (100nM) caused a statistically significant greater decrease in fEPSP (-53.4%) as compared to the CPA (100nM) response alone (**Figure 3.3.1 C,** where p=0.01).

On the other hand, when comparing between th**e** fEPSP responses following CPA (100nM) treatment, we found that at the end of the 45-minute washout period there was no statistically significant difference in fEPSP response when metformin (5μM)+CPA (100nM) was used as compared to CPA (100nM) alone (**Figure 3.3.1 D**, where p=0.7).

3.3.2. Effect of metformin on indiscriminate cell death during adenosine release

Our earlier electrophysiological finding (**Figure 3.3.1 C**) was an interesting finding which allowed us to investigate the possibility of metformin (5μM) exacerbating cell death in the presence of CPA (100nM).

Therefore, mimicking our CPA (100nM) electrophysiology study, we treated hippocampal slices with DMSO, CPA (100nM), metformin (5μM) and metformin (5μM)+CPA (100nM) and added propidium iodide staining to observe the levels of cell death. This would allow us to ascertain whether metformin treatment during transient adenosine A1 receptor stimulation (100nM of CPA for 30 min) is neuroprotective or not. Our results are shown as follows in **Figure 3.3.2 A and B**.

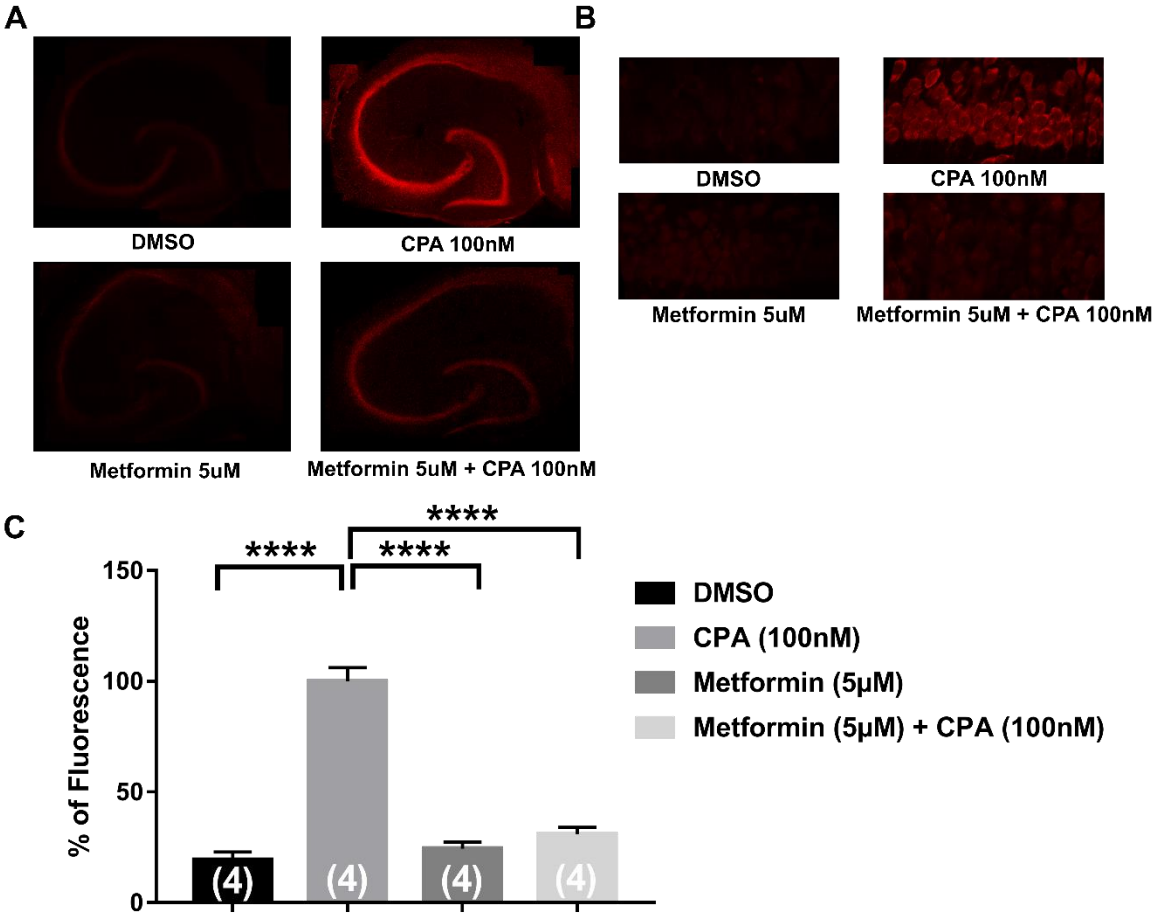

Figure 3.3.2: Metformin (5µM) preincubation prevented CPA-induced cell death. N=4 individual slices per treatment group.

Hippocampal slices were preincubated with metformin (5µM) or DMSO for 1 hour. CPA at 100nM was added to the required treatment groups accordingly for 30 minutes. All drugs were subsequently washed out with untreated aCSF for 2 hours, before propidium iodide staining was used in the final hour to quantify the overall levels of cell death via intensity of fluorescence. We quantified this fluorescence using densitometry, and analysed our results using statistical one-way ANOVA testing in **Figure 3.3.2 C** where $p<0.05$=*, $p<0.01$=**, $p<0.001$=***, $p=0.0001$=****.

A statistically significant decrease in propidium iodide red fluorescence was observed with metformin preincubated slices that were treated with CPA at 100nM (**Figure 3.3.2 C**). Interestingly, treatment with metformin alone did not induce any significant neuronal damage,

and fluorescence levels were very similar to DMSO control (p=0.81) or combined CPA and metformin treatments (p=0.68).

3.4. The effect of metformin on post-stroke cognition, mood and locomotion after chronic CPA administration

In the literature, one study noted that chronic metformin exposure 24 hours after MCAO led to increased neurogenesis, angiogenesis and cell survival following stroke (Jin et al., 2014). They attributed this neuroprotection to be mediated by adenosine monophosphate activated protein kinase (AMPK).

Based on our *ex vivo* findings and reports in the literature we wanted to investigate the effect of metformin *in vivo* with chronic supraphysiological increase of adenosine levels similar to what would be expected following stroke; so, we intraperitoneally injected animals with CPA (A1R agonist) at 3mg/kg for 7 days.

With such variations in metformin dosing within the literature ranging from 25mg/kg to 500mg/kg we decided to use 3 concentrations (2mg/kg, 5mg/kg, 10mg/kg) in our study. We chose these concentrations of metformin because our animals were not T2D sufferers and we did not want to prematurely induce lactic acidosis (metformin's primary adverse effect) in our animals. The maximum recommended therapeutic dose for T2D patients was 2,550mg/day and for a 70kg adult male this was calculated to be between 35-40mg/kg/day.

Our control was DMSO/Saline because all drugs were dissolved in DMSO first and then diluted with phosphate buffer saline to the appropriate volume.

3.4.1. Metformin at 10mg/kg significantly improves CPA-induced cognitive deficits (Y-Maze)

First, we used the Y-Maze behavior test to assess the effect of adenosine and metformin on hippocampal dependent spatial memory. We know there are large subpopulations of adenosine receptors within the hippocampus and most of the cognitive deficits observed following stroke often originate from neuronal damage in this region of the brain. Using the

knowledge gained from our earlier *ex vivo* results, we knew that metformin binds adenosine receptors and interferes with their signalling mechanisms to confer neuroprotection. Specifically, we found that metformin (5μM) preincubation prevented APSP generation (**Figure 3.2.1 D**), and neuronal cell death following A1R stimulation (**Figure 3.2.1 C** and **3.3.2 C**). Therefore, we expected to see significant improvements in cognition and functioning with metformin treatment after CPA-induced chronic insult which we knew to significantly affect the hippocampus (Stockwell et al., 2017). Our results are shown in **Figure 3.4.1**.

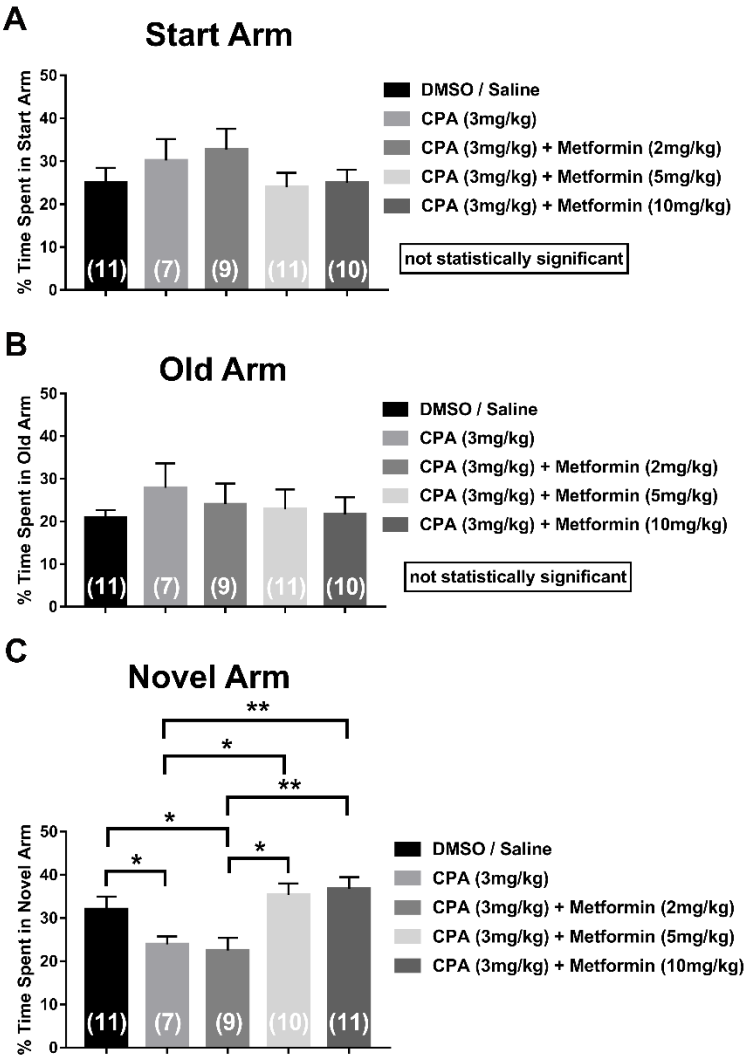

Figure 3.4.1: Rats treated with CPA at 3mg/kg and Metformin at 10mg/kg concurrently showed the greatest cognitive improvement as compared to rats treated with metformin at 2mg/kg and CPA at 3mg/kg or CPA at 3mg/kg alone. N=11 individual rats per treatment

groups – DMSO/Saline, CPA (3mg/kg)+Metformin (5mg/kg). N=7 individual rats per treatment groups – CPA (3mg/kg). N=9 individual rats per treatment group – CPA (3mg/kg)+Metformin (2mg/kg). N=10 individual rats per treatment groups - CPA (3mg/kg)+Metformin (10mg/kg).

Rats were treated with CPA alone (**3**mg/kg), CPA and Metformin at **2**mg/kg, **5**mg/kg and **10**mg/kg or DMSO/Saline once daily for 7 days. Behavior testing was conducted on day 8. Animals were allowed to explore two arms of the Y-maze for 15 minutes, removed from the maze and given 1.5 hours to consolidate any information learned to spatial memory. Following this, animals were placed back in the Y-maze, with all three arms of the maze open for 5 minutes. The percentage of time each animal spent in each of the three arms during this period was analysed using a one way-ANOVA test.

Y-Maze Testing for hippocampal-dependent spatial memory and learning showed CPA (3mg/kg) and CPA (3mg/kg)+Metformin (2mg/kg) treated rats had cognitive deficits significantly below that of DMSO/Saline, CPA (3mg/kg)+Metformin (5mg/kg) and CPA (3mg/kg)+Metformin (10mg/kg); with CPA (3mg/kg)+Metformin (10mg/kg) producing the greatest improvement in spatial memory acquisition and cognitive functioning. Statistical testing was performed using One-way ANOVA and Tukey post-test where $p<0.05$ =*, $p<0.01$=** (**Figure 3.4.1 C**). Y-maze testing was typically done in the morning prior to other behavioral testing performed later on the same day.

3.4.2 Metformin appears to improve CPA-induced locomotion deficits and anxiety

Open Field

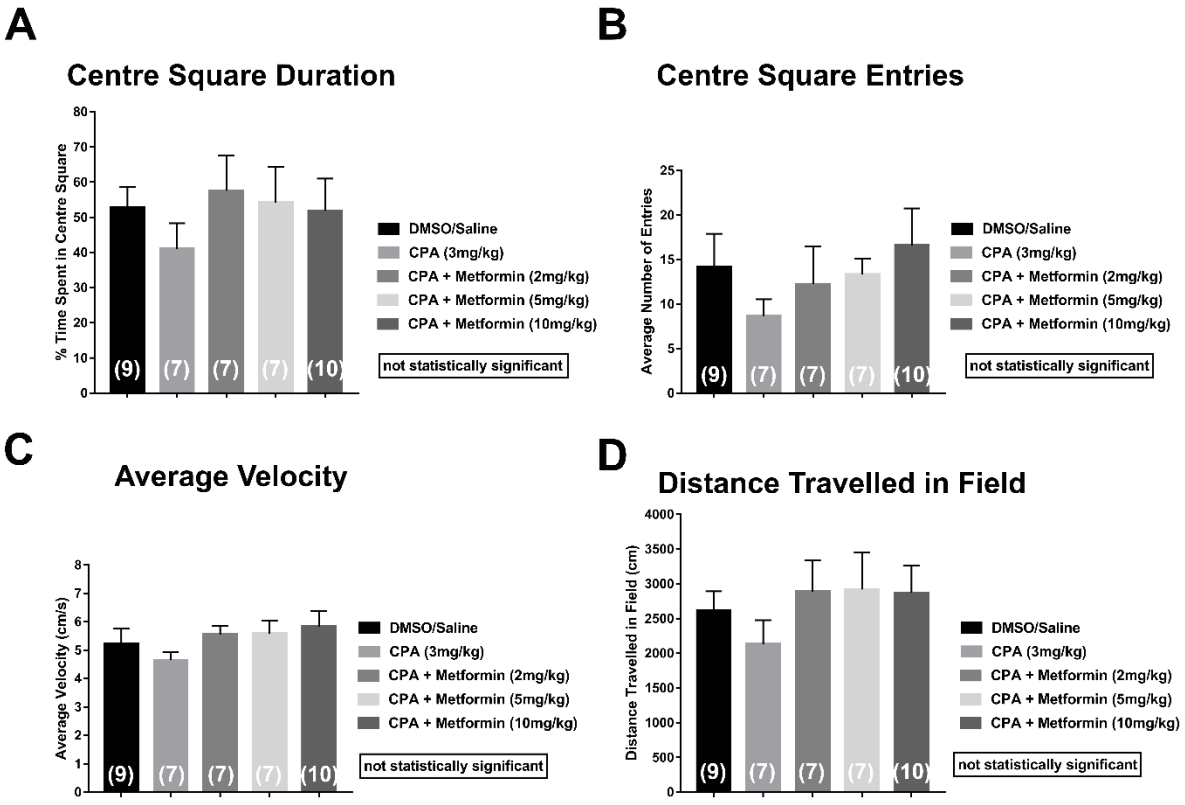

Figure 3.4.2: Metformin treatment at 2mg/kg, 5mg/kg and 10mg/kg appears to alleviate CPA-induced anxiety and improves exploratory behaviors. N=7 individual rats per treatment groups – CPA 3mg/kg, CPA+Metformin 2mg/kg, CPA+Metformin 5mg/kg. N=9 individual rats per treatment groups – DMSO/Saline, CPA+Metformin 10mg/kg. Extreme outliers were excluded from this data set.

Animals were placed in an open field maze, with an illuminated centre square, which was shown to induce anxiety and fear-like behaviors in prey animals leading to a preferential bias for frequenting the borders of the field versus the centre square. Animals were left to explore this field for 10 minutes, this data was analysed and one-way ANOVA and Tukey post-hoc statistical testing was performed.

DMSO/Saline and metformin treated animals at 2mg/kg, 5mg/kg and 10mg/kg spent a greater amount of time exploring the centre square (**Figure 3.4.2 A** and **B**) with an increased number of entries (**Figure 3.4.2 B**) and a greater duration of time spent in this area of the field (**Figure 3.4.2 A**), as compared to CPA-treated animals.

CPA treated animals appeared to frequent the borders of the maze (not shown) and had very few entries into the centre square (**Figure 3.4.2 A**).

Metformin treatment at 2mg/kg, 5mg/kg and 10mg/kg when given in conjunction with CPA at 3mg/kg seems to improve locomotion and exploration (**Figure 3.4.2 C and D**), with animals travelling a greater distance overall within the field (**Figure 3.4.2 D**). Although this difference is not statistically significant ($p=0.62$), a trend is apparent, however, more animals will be needed for testing to improve the statistical power of this test. As well as this, the average velocity of metformin-treated animals was increased as compared to CPA only treated animals which had the lowest overall velocity during the test (**Figure 3.4.2 C**). Again, this difference was not statistically significant ($p=0.37$), therefore, more animals will be needed for testing to improve the statistical power of this test.

Overall, the open field test revealed that CPA treatment caused increased anxiety and decreased mobility and the overall exploration of the field in these animals. However, metformin treatment at all three doses appeared to improve both anxiety and locomotion, although this result is not yet statistically significant, so no conclusions can be drawn from this test.

3.4.3 Metformin improves CPA-induced locomotion deficits and depression

Forced Swim

Following the Y-maze test (typically performed in the afternoon on the first day of behavior testing), a second behavior test was conducted on these animals to investigate the effect of adenosine and metformin on motor functioning. While limited in function, we used the forced swim test to compare how well the animal was able to swim (success) and how well the animal was able to keep its head above water (vigor), and how often the rat was immobile during the 10-minute trial.

3.4.3.1. Immobility

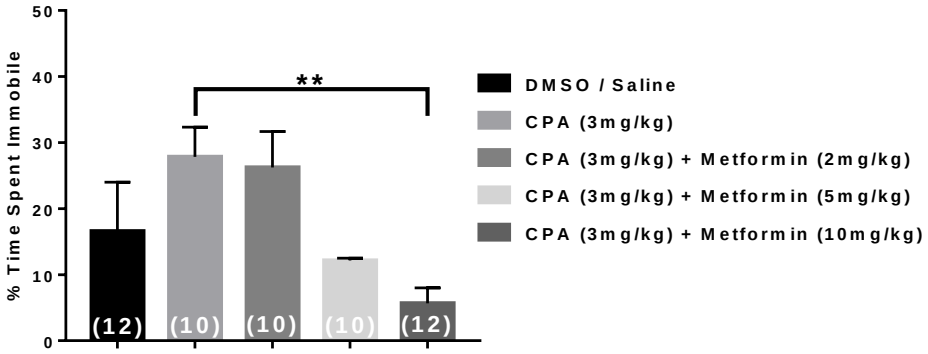

Figure 3.4.3.1: Metformin treatment at 10mg/kg showed significant increase in mobility during the trial as compared to CPA only treated rats which frequently spent long periods of time immobile in the water. N=10 individual rats per treatment groups – CPA 3mg/kg, CPA+Metformin 2mg/kg, CPA+Metformin 5mg/kg. N=12 individual rats per treatment groups – DMSO/Saline, CPA+Metformin 10mg/kg.

The length of time the rats appeared immobile (defined as no movement for at least 3 seconds) is positively correlated with depressive behavior, with greater periods of immobility being associated with greater levels of depression in animals.

CPA treatment produced increased % time of immobility (i.e., increased depressive behavior), whereas CPA +Metformin treatment at 10mg/kg appeared to attenuate depressive behaviors caused by CPA administration. One-way ANOVA statistical testing was performed where $p<0.05$=*, $p<0.01$=** and $p<0.001$=***.

3.4.3.2. Success

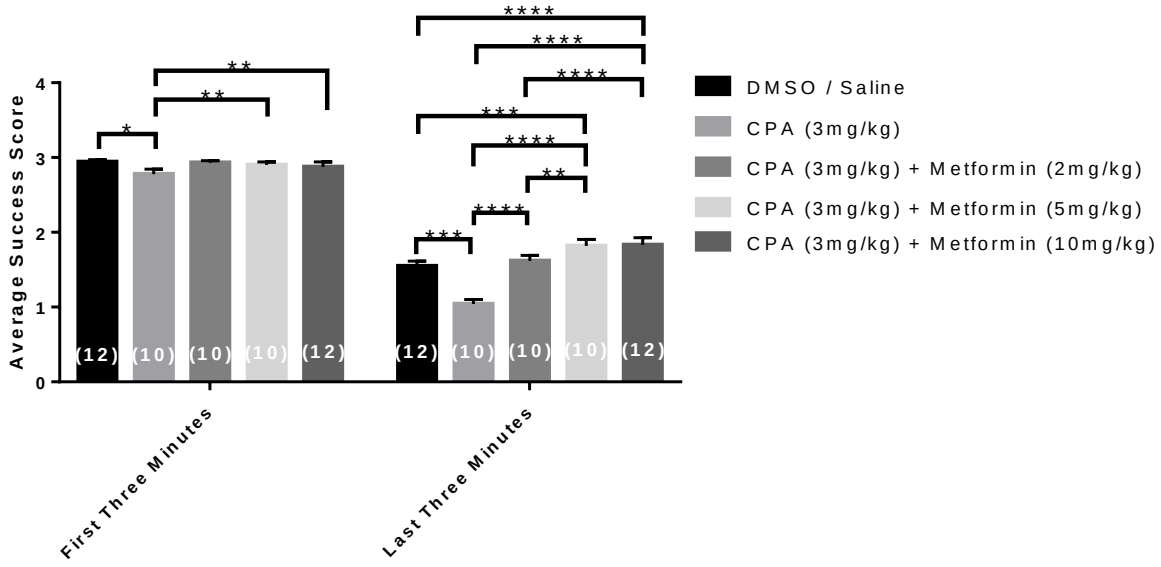

Figure 3.4.3.2: Rats treated with Metformin at 2mg/kg, 5mg/kg and 10mg/kg showed greater success scores than rats treated with CPA (3mg/kg) alone. N=10 individual rats per treatment groups – CPA 3mg/kg, CPA+Metformin 2mg/kg, CPA+Metformin 5mg/kg. N=12 individual rats per treatment groups – DMSO/Saline, CPA+Metformin 10mg/kg.

Rats were treated with CPA alone (3mg/kg), CPA and Metformin at 2mg/kg, 5mg/kg and 10mg/kg or DMSO/Saline once daily for 7 days. Behavior testing was conducted on day 8. Animals were placed in a tank filled with water at 25 degrees Celsius and left to swim for 10 minutes. Success scores were defined as follows: 3-Continuous movement of 4 limbs, 2.5-Occasional floating, 2-Floating more than swimming, 1.5-Ocassional swimming using 4 limbs, 1-Ocassional swimming using hind limbs only. 0-No use of limbs. The scores from the first three minutes of and the last three minutes of the trial were averaged and analysed using a one way-ANOVA test, where $p<0.05$=*, $p<0.01$=**, $p<0.001$=*** and $p<0.0001$=****.

Forced Swim testing for motor deficits showed CPA (3mg/kg) rats had the lowest scores for success **(Figure 3.4.3.2)** during the swimming period, when the first three minutes of testing were compared with the last three minutes of testing. Metformin treatment at all three doses was able to significantly alleviate CPA-induced motor deficits ($p=0.0001$).

3.4.3.3. Vigor

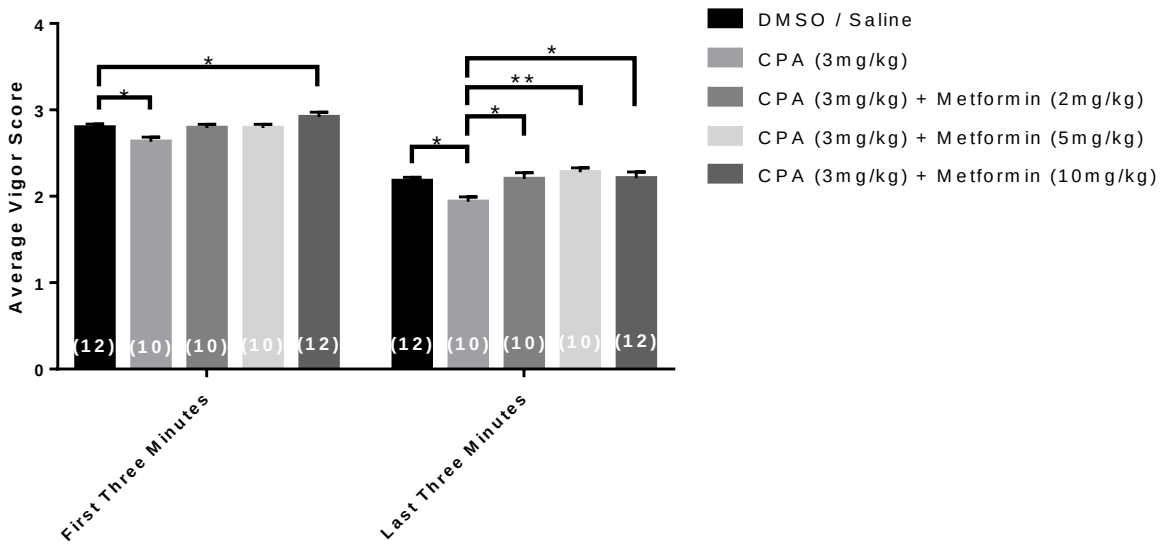

Figure 3.4.3.3: Rats treated with Metformin at 2mg/kg, 5mg/kg and 10mg/kg showed greater vigor scores than rats treated with CPA (3mg/kg) alone. N=10 individual rats per treatment groups – CPA 3mg/kg, CPA+Metformin 2mg/kg, CPA+Metformin 5mg/kg. N=12 individual rats per treatment groups – DMSO/Saline, CPA+Metformin 10mg/kg.

Rats were treated with CPA alone (3mg/kg), CPA and Metformin at 2mg/kg, 5mg/kg and 10mg/kg or DMSO/Saline once daily for 7 days. Behavior testing was conducted on day 8. Animals were placed in a tank filled with water at 25◦C and left to swim for 10 minutes. Vigor scores were defined as follows: 3-Entire head above water, 2.5-Ears but not eyes usually below water, 2-Eyes but not nose usually below water, 1.5-Entire head below water for >3 seconds, 1-Entire head below water for >6 seconds, 0-Animal on bottom of tank for periods of 10 seconds or longer. The scores from the first three minutes of and the last three minutes of the trial were averaged and analysed using a one way-ANOVA test, where $p<0.05$=* and $p<0.01$=**.

Forced Swim testing for motor deficits showed CPA (3mg/kg) rats had the lowest scores for success (**Figure 3.4.3.1**) and vigor (**Figure 3.4.3.2**) during the swimming period, when the first three minutes of testing were compared with the last three minutes of testing. Metformin treatment at all three doses was able to significantly alleviate CPA-induced motor deficits.

With regards to locomotion, we had previously shown in **Figures 3.4.2 C** and **D** during open field testing, that metformin improved exploratory behaviors and locomotion during open field testing. However, this finding was not statistically significant. On the other hand, with forced swim testing we were able to confirm in **Figures 3.4.3.1** and **3.4.3.2** that metformin treatment was able to improve locomotion. Overall it appears that all doses of metformin were sufficient to improve CPA-induced motor deficits, but metformin 10mg/kg was the best dose for the improvement of both motor and cognitive functioning of these animals.

3.5. The effect of metformin during chronic adenosine stimulation on A1R expression

We then conducted Western blotting experiments with biotinylated tissue to assess the effect of adenosine receptor expression with metformin following chronic CPA treatment.

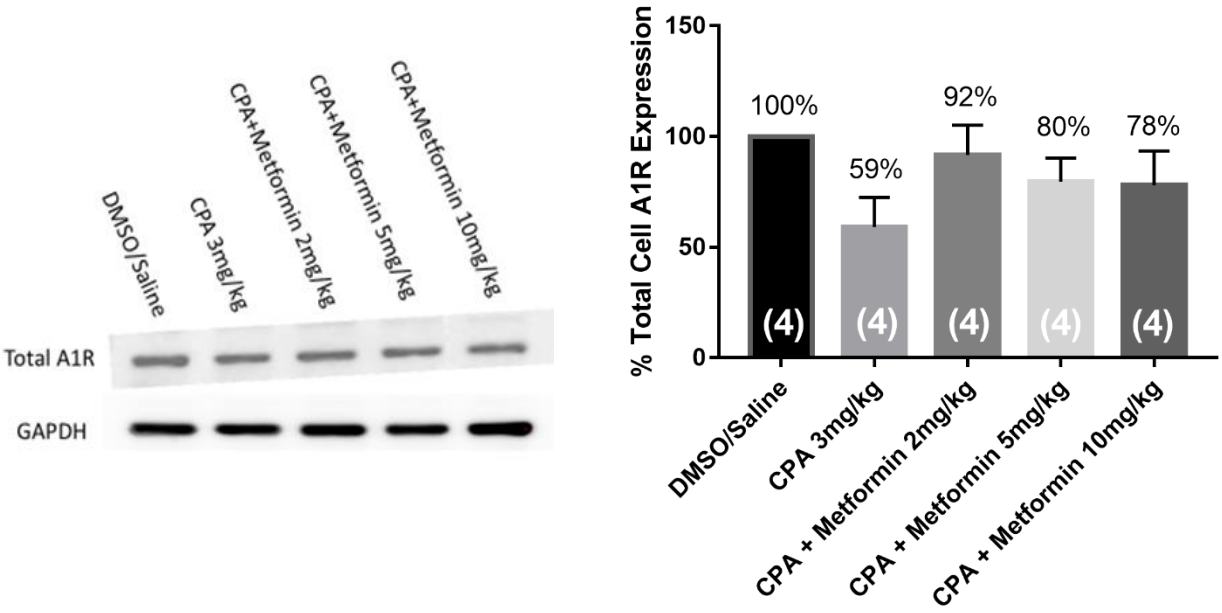

Figure 3.5: Metformin treatment appears to prevent the reduction in the expression of adenosine A1R in CPA-treated animals. N=4 with 3 individual animal samples per treatment group.

Hippocampal slices were taken from chronically injected animals, biotinylated and homogenised over ice with lysis buffer containing protease inhibitors to prevent degradation. This tissue was then centrifuged at 15,000g for 5 minutes, and the supernatant was removed and stored. Western blotting experiments were then carried out on this tissue with different

antibodies at 1:1000 concentrations. Blots were then imaged and analysed using Image J. Data was normalised to DMSO/Saline control values which show regular levels of A1R expression without the effect of CPA or metformin, and these values were averaged and then compared between treatment groups. One-way ANOVA statistical testing was performed, and the differences in A1R whole cell protein expression was found to not be statistically significant, where $p=0.16$.

Data so far suggests that CPA at 3mg/kg causes internalisation of A1R, while metformin treatment at all three concentrations (2mg/kg, 5mg/kg and 10mg/kg) restores or prevents the internalisation of A1R. However more tissue samples will need to be collected and added to this data set before any conclusions can be drawn from this experiment. Future studies will investigate whether metformin can also prevent alterations in the surface expression of GluA1 and GluA2 AMPARs as well as A1Rs and A2ARs. We will investigate this theory further with Western blotting experiments looking at both whole cell and surface receptor expression.

3.6. Metformin competitive adenosine A1 receptor binding in the presence of H^3-DPCPX

Radioligand Binding Assay

We conducted a radioligand binding assay using hot-DPCPX and cold metformin on hippocampal tissue in collaboration with Dr. Robert Laprairie from the College of Pharmacy and Nutrition at the University of Saskatchewan.

1nM [H^3] DPCPX, 2 h incubation at room temperature, 125 µg protein from rat hippocampus. Mean ±SEM, % relative to 100% DPCPX bound. n=8

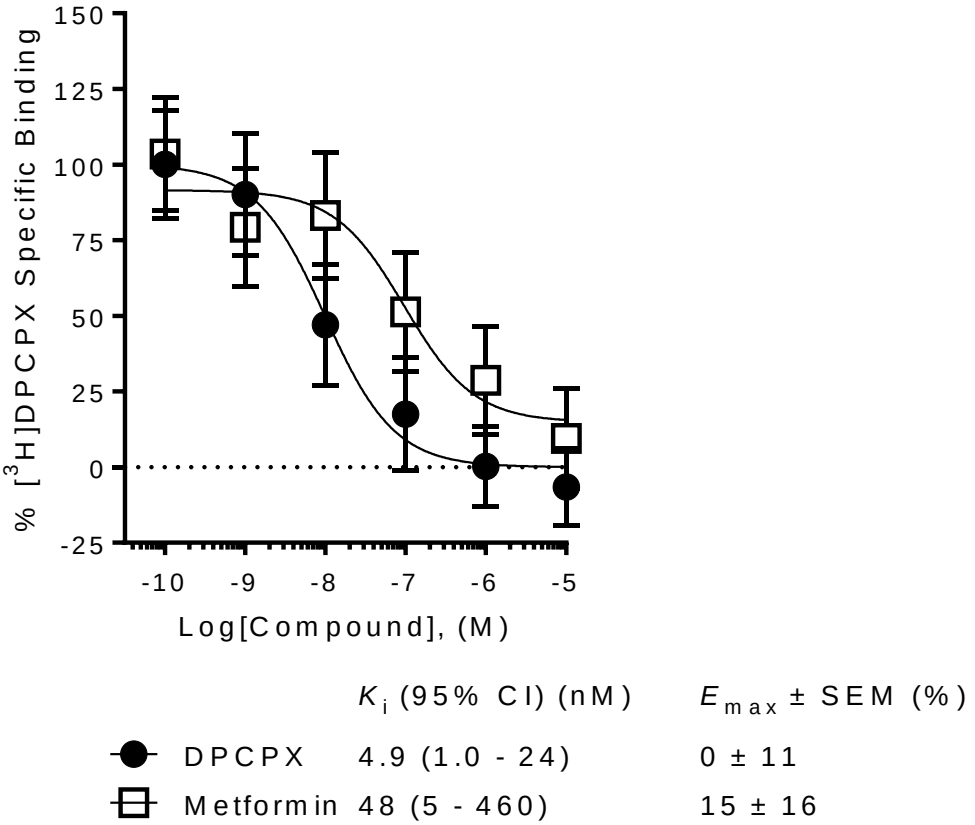

Figure 3.6: Metformin displaced adenosine A1 receptor binding by DPCPX. Metformin binds the adenosine A1 receptor with K_i of 48nM. K_d is a measure of the affinity of the ligand for its binding site. Since K_d is the concentration at which the ligand binds to half its binding sites, IC_{50} is the concentration of the inhibitor causing 50% inhibition of binding. K_i is calculated using IC_{50} and is defined as the inhibitor constant. N=8 individual age-matched sex-matched hippocampal samples from the same animal litter.

Hippocampal slices were rapidly frozen in liquid nitrogen, homogenised and centrifuged at 26500rpm to separate the cell membrane from the intracellular contents. Radioactive DPCPX (Perkin Elmer) was then used to competitively bind cold/non-radioactive DPCPX and cold/non-radioactive metformin at 6 different concentrations (0.1nM, 1nM, 10nM, 100nM, 1µM, and 10µM). The overall binding affinity of metformin and DPCPX to A1R was calculate in **Figure 3.6**.

Radioligand binding experiments yielded some interesting results suggesting that metformin binds to A1R with a binding constant (K_i) of 48nM, which is somewhat close to the binding affinity of adenosine for A1R reported in the literature to be at 70nM approximately. Our DPCPX binding studies revealed an affinity of 4.9nM for A1R with the literature reporting a value of log 8.4-9.2 which would approximately be between 1nM-10nM. These results did confirm our initial hypothesis concerning metformin A1R-binding but did not provide any information regarding the binding of metformin to any other adenosine receptors.

4. DISCUSSION

Adenosine receptor agonism and antagonism have been implicated in both exacerbating and ameliorating disease. As mentioned earlier, the effect of adenosine receptor activation depends on the concentration of adenosine present, and the subtype of adenosine receptor that is being primarily activated. The effects of A1R activation via CPA, and its potential inactivation via metformin, will be compared and contrasted with respect to the studies carried out and the results observed. These results will be discussed objectively in relation to the current body of literature surrounding the neuroprotective potential of metformin during stroke.

The effect of metformin on field excitatory post-synaptic potentials

Initially our findings from electrophysiological studies showed that metformin increases hippocampal synaptic transmission, at concentrations greater than 1μM (**Figure 3.1 C**). This was our first indication that metformin may act to influence A1/A2AR signalling mechanisms, as A1R is the most abundant adenosine receptor subtype in the hippocampus and is closely followed by adenosine A2AR.

We hypothesised that metformin could increase neuronal excitability and enhance the probability of neurotransmitter release at presynaptic nerve terminals by initially binding to A1R as an antagonist like DPCPX.

In C6 glioma cell lines that underwent hypoxic treatment, researchers found a downregulation of A1R and an upregulation of A2AR that was later reversed when A1R antagonist DPCPX was used, but not when A2AR antagonist ZM 241385 was used (Castillo, León, Ruiz, Albasanz, & Martín, 2008). Chen et al. (Z. Chen et al., 2014), also observed a downregulation of A1R and an upregulation of A2AR in an *in vivo* focal cortical ischemia model (using the pial vessel disruption or PVD stroke model), which suggests that A2AR expression is dependent on A1R activation.

These earlier studies supported our current findings. First, consistent with metformin binding and inhibiting A1Rs, we found that metformin binding reduces the inhibitory tone on baseline synaptic transmission which is normally a reflection of actions of endogenous levels of

adenosine on A1Rs. We observed enhanced fEPSPs in CA1 pyramidal neurons in the hippocampus when doses greater than 1μM of metformin were tested for periods of 15 minutes or longer. Second, we observed that metformin prevented the A1R-dependent upregulation of A2AR excitatory activity, as the A2AR-dependent hippocampal synaptic potentiation was never observed during prolonged normoxic washout. Third, we found that surface expressed A1Rs were downregulated in the presence of the A1R agonist CPA (Z. Chen et al., 2014; Z. Chen, Stockwell, & Cayabyab, 2016), and this was largely prevented by metformin. Fourth, behavioral deficits (cognitive dysfunction, depression and anxiety-related behaviors) observed after chronic CPA treatments and attributed mainly to the persistent A1R stimulation by CPA, were abrogated by metformin, similar to the effects seen with the A1R antagonist DPCPX. Finally, based on our observations with high concentrations of metformin at 30μM where there was a sudden decrease of fEPSP upon perfusion, we speculated that metformin at such high concentrations may be showing non-specific binding to other targets other than adenosine receptors. Whether metformin effects at higher concentrations hinders the neuroprotective effects observed at lower metformin concentrations, presumably due to actions on other molecular and cellular targets, remains to be established.

The effect of metformin during hypoxic insult

Now, in relation to the thesis topic at hand which looks to investigate the role of adenosine A1 receptor action in stroke pathophysiology, we know that A1R activation decreases calcium influx (Wu & Saggau, 1997) and supresses the release of excitatory neurotransmitters like glutamate (Andiné, Rudolphi, Fredholm, & Hagberg, 1990; Corradetti, Lo Conte, Moroni, Passani, & Pepeu, 1984; Fastbom & Fredholm, 1985) acetylcholine (Spignoli, Pedata, & Pepeu, 1984) and dopamine (Chowdhury & Fillenz, 1991; Michaelis, Michaelis, & Myers, 1979). In addition, A1R activation has also been shown to suppress the release of the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) (Hollins & Stone, 1980) (Stone, Ceruti, & Abbracchio, 2009).

When high concentrations of adenosine (10-300μM) were utilised, like those that would be found during hypoxic insult, A1R activation appears to have a greater effect in supressing glutamate release than in suppressing GABA release to exert its inhibitory effect (Hollins & Stone, 1980; Lucchi, Latini, de Mendonça, Sebastião, & Ribeiro, 1996). This preferential bias of A1Rs in decreasing glutamate release over GABA when mediating its inhibitory effect is

believed to limit the chance for A1R antagonists to cause rebound hyperexcitability (Stone et al., 2009), which would result in the increase of seizure-like activity as the inhibition which normally would regulate this excitability would have been completely lost.

This hypothesis although confirmed later by Stone and colleagues (Stone et al., 2009), was initially proposed and studied by Von Lubitz and colleagues (D. K. J. E. Von Lubitz, Paul, Ji, Carter, & Jacobson, 1994). In their study, chronic A1R antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX) treatment eliminated NMDA-induced clonic-tonic seizure episodes, increased average survival time and decreased end point mortality after NMDA administration but chronic A1R agonist $N^{(6)}$cyclopentyladenosine (CPA) treatment did not, and rather potentiated the NMDA-induced effects.

On the other hand, acute treatment with CPA resulted in the delay or elimination of seizure onset and a significant reduction in mortality, while acute CPX treatment lead to mortality (D. K. J. E. Von Lubitz, Paul, Carter, & Jacobson, 1993).

These studies investigated the effects of acute and chronic A1R agonists and antagonists on NMDA-evoked seizures, and found chronic A1R antagonism and acute A1R agonism to be neuroprotective and synonymous in nature, thereby alluding to their actions potentially being mediated via the same A1R secondary messenger systems (D. K. J. E. Von Lubitz et al., 1994).

It is known that adenosine elevation during hypoxia results in the activation of adenosine A1R receptors, leading to persistent synaptic depression via decreased calcium and glutamate release. We proposed that if metformin acts as an A1R antagonist, it should prevent the hypoxia-induced synaptic depression like DPCPX does to confer neuroprotection.

Interestingly, in our experiments we observed a time-dependent neuroprotective effect of metformin during hypoxia where metformin at 5μM perfused 5 minutes into hypoxia was not neuroprotective but preincubation with the same concentration of metformin prior to the induction of hypoxia resulted in significantly reduced cell death as compared with hypoxic controls (**Figure 3.2.2**).

On the other hand, when we analyzed our electrophysiological data, we saw that metformin treatment did not prevent hypoxia-induced synaptic depression as we had initially

hypothesized. In fact, there was no significant difference between the synaptic depression observed between our control hypoxia treatment, metformin (5μM) when given 5 minutes into hypoxia treatment or metformin (5μM) preincubation prior to the induction of hypoxia treatment (**Figure 3.2.1 C**). Nevertheless, we observed metformin (5μM) still induced neuroprotection against hypoxia-induced cell death (**Figure 3.2.2 C**).

This was an unexpected finding because we believed metformin should have prevented this synaptic depression if it mainly acts as an A1R antagonist, but a literature search revealed that long term depression (LTD)-inducing stimuli, like hypoxia, or treatments with glutamate agonists or treatment with insulin, the antidiabetic agent, all were shown to promote the internalisation of AMPARs (Haas, 2001).

Insulin was shown to mediate AMPAR internalisation via a different mechanism than glutamate as insulin treatment caused the internalisation of GluA2, the calcium impermeable AMPAR subunit, but not GluA1, the calcium permeable AMPAR subunit. This was due to a mechanism that required the last 15 amino acids of the C terminus of GluA2 (Haas, 2001), although both mechanisms utilised dynamin-dependent clathrin-coated pits to mediate endocytosis. Therefore, our observation indicating that while metformin does not affect the A1R-mediated synaptic depression observed during hypoxia (**Figure 3.2.1 C**), it is possible that metformin may be contributing to the internalisation of AMPAR subunits as well as A1Rs during hypoxia independent of clathrin-mediated endocytosis. This latter idea regarding clathrin-dependent vs. clathrin-independent (or dynamin-independent) endocytosis of surface expressed AMPARs and adenosine receptors warrants further investigations.

Now based on other unpublished observations indicating the important contributions of calcium-permeable AMPARs in post-hypoxia synaptic potentiation (submitted Qin et al., 2019), if metformin is involved in AMPAR internalisation during hypoxia then it may also be involved in mediating the post-hypoxia re-insertion of GluA1/A2 AMPA receptor subunits, which we now know to be implicated in excitotoxic and pro-apoptotic pathways. The rapid and sustained increase in fEPSP above baseline following normoxic treatment after hypoxia was attributed to an adenosine-mediated phenomenon called APSP (adenosine-induced post-hypoxia synaptic potentiation); whereby adenosine A1 and A2A receptors mediate AMPAR trafficking and recycling, causing the reinsertion of the GluA1/A2 AMPA receptor subunits in to the cell surface membrane (Z. Chen et al., 2014; Stockwell et al., 2016).

(Stockwell et al., 2016) demonstrated that the reinsertion of AMPARs into the cell membrane following hypoxia resulted in neurotoxicity which was harmful to hippocampal cell health. Their studies showed that preincubation with protein phosphatase inhibitors (Stockwell et al., 2016) or the utilisation of certain AMPAR antagonists (submitted Qin et al., 2019) was able to prevent this AMPAR reinsertion and accompanying synaptic potentiation when administered either during hypoxia or post-hypoxia which resulted in significantly lower levels of cell death.

Therefore, our results suggest that post-hypoxic AMPAR recycling/re-insertion may depend on how metformin (5μM) preincubation prior to hypoxia was able to bind to and prevent the actions of adenosine A1 and A2A receptors, which are known to modulate the viability of cells within the hippocampus even though metformin was unable to prevent the hypoxia-induced synaptic depression (**Figure 3.2.1 B**).

Our electrophysiological results did in fact reveal that metformin (5μM) preincubation prevented the development of APSP while metformin 5μM treatment 5 minutes into hypoxia did not prevent APSP (**Figure 3.2.1 D**). Given that we know A1R and A2AR activation are both implicated in AMPAR recycling, we realised that there must be a time-dependent mechanistic change that was occurring at the level of the post-synaptic membrane which influences postsynaptic AMPARs (Haas, 2001) and adenosine receptor activity. (Z. Chen et al., 2014).

Qin and colleagues (submitted Qin et al., 2019), also showed that preincubation with antagonist of either A1R (DPCPX) or A2AR (SCH58261) prevented the full expression of APSP, and that an A1R- and A2AR-dependent insertion of GluA1 and GluA2 AMPAR subunits, may also be a potential target of a metformin-A1R-dependent interactions.

Additionally, it is also important to note the effect of metformin on mitochondrial function as it relates to hypoxia. We know from previous studies that mitochondria are very important for respiration where they work to generate most of the ATP that the cell requires. Now mitochondrial dysfunction on the other hand, has been correlated to a number of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), AD, PD and HD (Lin & Beal, 2006). In these conditions, mitochondrial dysfunction leads to an increase in the production of ROS (Lin & Beal, 2006). Under normal physiological conditions, ROS/free radicals are rapidly mopped up by oxygen molecules to form water via reactions involving

several enzymes present in the mitochondrial space (Lin & Beal, 2006). However under pathophysiological conditions this process is disturbed, and these ROS molecules cause irreparable damage to the cell leading to apoptosis (Lin & Beal, 2006; Rego & Oliveira, 2003). Now, in relation to hypoxia which we know depletes the level of oxygen available to the tissues, these free radicals/ROS could not be processed to less harmful compounds such as water. It is therefore reasonable to deduce that hypoxic conditions, such as those present in our electrophysiology and histology studies (**Figure 3.2.1** and **3.2.2** respectively), would subsequently trigger increases in ROS levels, causing the cell to favor proapoptotic pathways which would augment cell death.

Several studies within the literature have noted that metformin is able to inhibit complex I of the mitochondrial respiratory chain (Viollet et al., 2012). We know that complex I is the first of five complexes that make up the respiratory chain, so we believe that by inhibiting the first of these complexes, metformin is able to prevent the production of ROS, thereby rescuing tissues from ROS-related cell damage, but this concept warrants further investigation.

Overall, we believe that the disruption of adenosine receptor signalling mechanisms via metformin, and potentially its additional role in reducing mitochondrial ROS production are involved in mediating its neuroprotective capability to rescue cells from hypoxia-induced cell death.

The effect of metformin on supraphysiological adenosine increase

CPA is a potent adenosine A1R agonist, therefore we believed it would be a good compound to test the idea of metformin A1R antagonism using electrophysiology methods in hippocampal brain slices.

With CPA treatment at 100nM we observed a significant decrease in fEPSP after 10 minutes (**Figure 3.3.1 B**). This finding was expected as earlier studies had shown that a 50nM CPA administration for 10 minutes also caused significant levels of synaptic depression with fEPSP signalling almost recovering back to the baseline (Brust et al., 2007). Further studies showed that while 50nM CPA was sufficient to induce p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) activation, higher concentrations of CPA with longer

incubation times like 500nM CPA for 30-45 minutes also resulted in significant changes in surface expression of GluA1 and GluA2 subunits (Z. Chen et al., 2014).

Now given our hypotheses regarding metformin-A1R binding, we expected that metformin-treated hippocampal slices would resist CPA-induced synaptic depression, however, we were surprised by our results. When slices were preincubated with metformin (5μM) and then treated with CPA at 100nM, rather than observing a prevention in the development of synaptic depression, instead we observed the opposite, and saw a greater level of synaptic depression as compared to CPA alone (**Figure 3.3.1 C**). One possibility that could account for this further enhancement of synaptic depression observed in the presence of metformin and subsequent CPA, is that there may be significant differences in the binding affinity of metformin (see below) and CPA (IC_{50}=58nM (Lohse et al., 1988)) to adenosine receptors. That is, it is possible that the compound with the higher affinity for A1R (CPA) may be able to displace the lower affinity drug (metformin, see below) from binding to A1R. The higher levels of CPA-induced synaptic depression in the presence of metformin could be explained by CPA being able to displace metformin from A1R, so that more A1Rs could be occupied by CPA, thereby producing increased synaptic depression. Alternatively, it is plausible that the enhanced synaptic depression in the presence of CPA + metformin could also be related to metformin binding to and inhibiting A2ARs, thus further inhibiting A2AR-mediated presynaptic glutamate release. Hence, future studies are needed to clarify these potential novel effects of metformin on adenosine signalling and regulation of synaptic transmission in hippocampus and other regions of the brains.

Additionally, we thought that because there was a greater decrease in fEPSP signalling with metformin as compared to CPA 100nM treatment alone (**Figure 3.3.1 C**) we would also see increased propidium iodide fluorescence (i.e., more neuronal death) in this treatment group; however again we were surprised because metformin (5μM) treatment decreased the level of propidium iodide fluorescence which was indicative of decreased levels of cell death (**Figure 3.3.2 C**). This suggested that metformin's neuroprotective actions during CPA treatment could involve signalling pathways independent of A1R stimulation, that potentially may involve the blocking of A2AR which is pro-neurotoxic.

Now considering the implications of the results from both our acute CPA administration experiments (**Figures 3.3.1** and **3.3.2**) and hypoxia electrophysiological experiments with and

without metformin (**Figures 3.2.1** and **3.2.2**), we believe that metformin may be involved in regulating A1R-induced AMPAR internalization by blocking A1R activation and subsequent downstream signalling via p38 MAPK, JNK, and PP2A (Brust et al., 2007, 2006; Z. Chen et al., 2014). However, it is interesting to note that when electrophysiological effects of metformin were examined (**Figure 3.3.1 C**), we found evidence of synergistic effects of metformin and CPA (i.e., greater synaptic depression, consistent with greater A1R stimulation and possibly greater subsequent A1R internalization). This highlights the need to perform additional experiments to clarify the acute effects of metformin on CPA-induced synaptic depression. However, as discussed below (**Figure 3.5**), the changes associated with metformin and CPA co-administration in our chronic CPA *in vivo* studies are clear, i.e., metformin appears to prevent CPA-induced internalization of A1Rs. Additional experiments are required to assess the effects of metformin on acute (*ex vivo* slices treatments) or chronic (*in vivo* drug treatments) CPA treatments on A1Rs and AMPARs.

Furthermore, when we compared the effect of metformin after hypoxia washout, we saw that metformin preincubation did also prevent the development of post-hypoxia APSP (**Figure 3.3.1 D**). Therefore, taken together we believed that acute application of metformin may indeed be implicated in preventing A1R internalization. Additional studies should not only investigate metformin and A2AR binding, but also should address whether metformin also affects the subsequent AMPAR insertion to the cell surface after normoxic reperfusion, and whether this is the likely mechanism for the reduced cell death revealed in our propidium iodide fluorescence studies.

The effect of metformin on chronic adenosine stimulation

Considering our current theory concerning metformin potentially regulating AMPAR cycling after blocking of A1R binding, we decided to assess the effect of metformin *in vivo* using known potent A1R agonist, CPA. Previous studies, as mentioned earlier, revealed that A1R is implicated in regulating A2AR activity and in controlling AMPAR internalization (Z. Chen et al., 2014) and reinsertion into the cell membrane (submitted Qin et al., 2019), therefore CPA was the drug of choice for utilisation in our *in vivo* studies.

CPA is an interesting drug because its acute administration has been shown to exert neuroprotective effects during times of cellular stress and/or hyperexcitability and this is a

finding that has been noted in the literature in various studies (Melani, Pugliese, & Pedata, 2014; D. K. J. E. Von Lubitz et al., 1994; Wardas, 2002).

When investigating the potential effect of CPA administration *in vivo*, we found in one study in particular where researchers contrasted the effect of acute and chronic CPA and CPX treatment on spatial memory acquisition in C57BL/6 mice that underwent the Morris water maze trials (D. K. Von Lubitz, Paul, Bartus, & Jacobson, 1993). Although the dosing of CPA used in these earlier studies was several times lower than what was used in this thesis, it was interesting to note that their study found low chronic i.p. doses of CPA at 5μg/kg, 10μg/kg, 20μg/kg and 30μg/kg over 9 days resulted in a dose-dependent reduction in target latencies, rapid spatial preference development and in the absence of animals that were unable to perform the task. On the other hand, CPX treatment at 0.2mg/kg, 0.5mg/kg and 1mg/kg resulted in the opposite. Interestingly in animals given doses greater than 1mg/kg, locomotor disturbances were also apparent (D. K. Von Lubitz et al., 1993). This study was one of the first publications to suggest that low dose CPA was able to improve spatial memory formation.

In contrast, another study noted that the acute stimulation of adenosine A1 receptors via CPA at doses ranging from 0.15μmol/kg to 2.25μmol/kg caused a dose-dependent impairment in the acquisition and retention of memory during a lick suppression task which measured passive avoidance (Normile & Barraco, 1991). These studies highlighted significant differences in the effect of CPA at different concentrations and given that adenosine mediates a range of different functions, we knew that it was very important to find the limit of the therapeutic window for CPA dosing that we could safely utilise to induce neurotoxic cerebral effects without inducing other deleterious systemic effects.

Fortunately, a follow-up supporting study by (D. K. Von Lubitz et al., 1994) looked into the effect of acute (i.p. 15 minutes prior), chronic (i.p. 15 days prior), low-dose (0.02mg/kg) and high-dose (1mg/kg) CPA and CPX treatment on ischemic damage and survival after occlusion of the carotid arteries. These researchers found significant differences in their results which became the backbone for the interpretation of our data in the *in vivo* study.

Acute CPA treatment at 0.02mg/kg or 1mg/kg, significantly improved survival rate (94% and 90% respectively) and neuronal preservation in comparison to vehicle-controls (60%). While on the other hand, chronic CPA treatment at 0.02mg/kg resulted in no significant difference in

survival rate (74%) and neuronal preservation, and 1mg/kg chronic CPA treatment resulted in a significantly lower survival rate (14%), and fewer surviving neurons (D. K. Von Lubitz et al., 1994). Acute CPX treatment at 1mg/kg also resulted in significantly lower survival rates (20%) and decreased neuronal survival, while chronic CPX treatment at 1mg/kg resulted in increased survival rate (93%) and greater neuronal preservation.

Taken together these studies highlight that the duration and dose of A1R agonist and antagonist administration are equally important in modulating hippocampal damage, spatial memory formation, and survival rates after ischemia, contributing to the idea of ischemic preconditioning (Dunwiddie & Masino, 2001). To elaborate, acute A1R stimulation, or low dose chronic A1R stimulation is thought to confer neuroprotection against pathological events by increasing the inhibitory effect of A1R. However, there is also data suggesting that A1R agonists when given chronically at high doses are neurotoxic as they cause the internalisation of A1R and thus the loss of the A1R-mediated inhibitory neuroprotective effect that other studies had been reporting earlier. It appeared that concentrations of CPA greater than 1mg/kg were neurotoxic when administered chronically (D. K. Von Lubitz et al., 1994) or greater than 5mg/kg when administered acutely (Stockwell et al., 2017).

Consequently, in our study we had chosen to utilise 3mg/kg of CPA to be injected once daily over 7 days, to facilitate the CPA-induced neurotoxic effects that we hoped metformin would counteract as a proposed A1R antagonist. We realised that if metformin was able to prevent CPA-induced behavioral deficits and effects then there would be sufficient experimental evidence to suggest not only metformin-A1R binding but direct A1R antagonism as well.

The effect of metformin on learning circuits

With CPA at 3mg/kg treatment, we observed significant impairments in hippocampal-dependent spatial memory and learning during Y-maze testing which we attributed to be caused by A1R-mediated neurotoxic pathways. CPA treatment also caused significant damage to hippocampal neural circuits and augmented cell death in DG and CA1-3 areas but metformin treatment at 10mg/kg was able to significantly reverse this damage (data not shown) and restore cognitive function (**Figure 3.4.1**).

We know that CPA causes the internalisation of A1Rs (Z. Chen et al., 2014), and the loss of A1R has also been implicated in AD pathology and in dementia studies (Kalaria, Sromek, Wilcox, & Unnerstall, 1990). Radioactive DPCPX, a high affinity A1R antagonist, was used in another study to confirm or dispute the suggestion of A1Rs loss within the hippocampus being implicated in AD pathology and dementia (Deckert et al., 1998). This seemed to be a logical hypothesis to test given that there are large numbers of adenosine receptors in the hippocampus which is the centre for learning and memory. However, their study found A1R loss to also be implicated in several other pathologies and concluded that A1R loss was not specific to AD pathology (Deckert et al., 1998). Interestingly A1R knockout models did not show deficits in spatial memory formation (Giménez-Llort et al., 2005) suggesting that there may be compensatory mechanisms involved in hippocampal dependent memory/learning circuits that are independent of A1R.

Interestingly with low concentrations of metformin (2mg/kg), CPA administration had an increasingly negative effect on hippocampal spatial memory formation (**Figure 3.4.1**). One explanation for the reason behind this increased deficit goes back to the idea of adenosine agonism versus antagonism having contrasting effects; and given that the dose of metformin is less than the dose of CPA, we believed that it was not enough to counteract the CPA-induced cognitive deficits.

Within this same treatment group we also noted an increase in the proportion of time these animals spent not moving within, or frequently returning to the start arm of the Y-maze, versus the time they spent exploring the novel arm which could be indicative of anxiety (Montgomery, 1955). To further investigate this result, we performed the open field test.

The effect of metformin on anxiety behaviors

While all animals in our study showed a preferential bias for the borders of the maze, CPA-treated animals had the fewest number of entries into the centre of the field (**Figure 3.4.2 B**). They also frequently exhibited freezing behavior throughout the trial and had the slowest overall velocity (**Figure 3.4.2 C**). On the other hand, metformin-treated animals at all concentrations explored the maze and showed an approximately equal increase in the number of entries into and time spent in the centre square over CPA-treated animals (**Figure 3.4.2 B** and **3.4.2 A** respectively). Similarly, A1R-knockout mice also showed reductions in

exploratory behavior in the open field test, and increased anxiety in the plus maze and dark-light box (Giménez-Llort et al., 2002).

It was also noted by Giménez -Llort and colleagues (Giménez-Llort et al., 2002), that A1R-knockout mice exhibited reduced muscle strength. Therefore, given that our open field test results had revealed that CPA-treated animals had lower velocities (**Figure 3.4.2 C**), travelled less (**Figure 3.4.2 D**) and spent a larger proportion of their exploration time immobile in the field, as well as the fact that we knew CPX-treatment at 1mg/kg caused locomotor changes (D. K. Von Lubitz et al., 1993), we decided to utilise the forced swim test to investigate whether CPA treatment at 3mg/kg caused motor changes and/or deficits.

The effect of metformin on depressive behaviors

The forced swim test revealed less successful (**Figure 3.4.3.2**) and vigorous (**Figure 3.4.3.3**) swimming in CPA-treated animals as compared to controls. These animals failed to keep their heads above the water during the trial, often did not utilise all their limbs to improve swimming ability and spent large periods of time immobile in water. This could be due to CPA-mediated changes in the basal ganglia direct pathway or it could be due to an increase in fear and depressive behaviors which would also cause these same effects. Further experiments like the sucrose-preference test or even the imaging of dopaminergic neuronal pathways would be a great way to assess this result.

Interestingly, all concentrations of metformin improved CPA-induced motor deficits. Metformin-treated animals spent most of their time mobile in water, dived frequently within the tank and swam successfully throughout the trial. Earlier in this thesis it was mentioned that metformin treatment at 2mg/kg was not sufficient to ameliorate the CPA-induced learning deficit that was observed during Y-maze testing (**Figure 3.4.3.1**). This dose of metformin in combination with CPA at 3mg/kg was shown to result in poorer cognition and an increase in anxiety-like behavior. However, during the forced swim test within this same treatment group we observed significant improvements in CPA-mediated depressive behavior which was an unexpected finding given the previous Y-maze testing result. This led us to question whether depressive circuits were less sensitive to stress and injury than hippocampal learning circuits.

A study by Snyder and colleagues (Snyder, Soumier, Brewer, Pickel, & Cameron, 2011) showed that the inhibition of neurogenesis in the hippocampi of adult mice increased despair-like behaviors. Therefore given that several studies have confirmed the ability of metformin to induce neurogenesis in the brain (Ghadernezhad et al., 2016; Liu, Tang, Zhang, et al., 2014; Patil, Jain, Ghumatkar, Tambe, & Sathaye, 2014; J. Wang et al., 2012), it is very possible that the dose of metformin utilised was too low to be beneficial in improving CPA-induced memory deficits, and that this was the reasoning behind that result. Indeed, this finding may highlight a novel mechanism in the pathway to understanding metformin-adenosine interactions, but other experiments are needed, and this may be a future direction for this study.

Taken together, our behavioral experiments revealed that 10mg/kg of metformin co-administered with CPA 3mg/kg was the most effective dose *in vivo* in conferring neuroprotection against CPA-induced cognitive and locomotor deficits. Metformin at this dose also improved hippocampal cell health and prevented the development of anxiety and depressive behaviors.

Metformin binding to adenosine receptors

Another important finding in our study was verifying the novel interaction for metformin binding to A1R. Western blotting studies confirmed earlier results by Chen et al. (Z. Chen et al., 2014) suggesting that prolonged A1R stimulation via CPA administration causes the internalization of A1R. Our results also showed that metformin treatment at all three concentrations appeared to prevent this internalization (not shown), although this difference was not statistically significant. Additionally, radioligand binding studies using H^3-DPCPX revealed that metformin binds to A1R with an affinity of 48nM, making it an A1R ligand. To compare the binding affinity of metformin with other A1R ligands to know its relative potency, we utilised non-radioactive DPCPX as our control which revealed a 4.9nM binding affinity for A1R (**Figure 3.6**). However, radioligand binding could be utilised with other A1R agonists and antagonists like 2-Chloro-N^6-cyclopentyladenosine (CCPA), or rolofylline (KW 3902) (Kenneth A Jacobson & Gao, 2006) to better characterise the metformin-A1R binding profile.

While radioligand binding studies reveal a novel metformin-A1R binding interaction, which is supported by our previous experiments both *ex vivo* and *in vivo*, we have to note that we did also observe an A2AR antagonist-like response during our hypoxia electrophysiology studies

(**Figure 3.2.1 B**). This means that we cannot rule out the possibility that metformin has non-specific adenosine receptor binding potential, which it utilises to mediate its neuroprotective effect. We know from the literature that adenosine receptors have been shown to interact with other adenosine receptors to form heteromers (Ciruela et al., 2006), and the same observation has also been noted with dopamine receptors (Fuxe, Ferré, Genedani, Franco, & Agnati, 2007). Therefore, there is also a possibility that metformin may bind A1R specifically but mediate the A2A receptor response via this mechanism.

All these ideas and concepts are significant to unravelling the mystery behind the role of metformin in improving T2D/stroke patient outcome because it would explain why a number of T2D sufferers treated with long-term metformin have lower rates of PD (Wahlqvist et al., 2012) and stroke (Cheng et al., 2014). Although with regards to metformin and AD, there is some controversy as to whether metformin ameliorates or exacerbates the condition (Imfeld, Bodmer, Jick, & Meier, 2012; Yarchoan & Arnold, 2014). On the other hand, in pathologies where we know A1R is implicated in the development of the disorder, utilising metformin could yield significant therapeutic benefits.

5. LIMITATIONS

Some limitations of our study were that we used *ex vivo* hypoxia treatment as an alternative means of reproducing some responses to an ischemic stroke on hippocampal brain slices. Not only did we change the environmental conditions of the brain during the decapitation and slicing procedure but we also could not mirror the pathophysiology of stroke using hypoxia. Another limitation of the study was that we did not utilise an *in vivo* stroke model to confirm our findings, as chronic A1R stimulation via i.p. injections also would not mimic pathological stroke conditions. Another limitation of the study is that we did not utilise other radioactive and non-radioactive adenosine A1R ligands to compare and contrast metformin A1R-binding affinity with other ligands. It would have been interesting to test the effect of metformin administration on blood glucose levels to determine if any of our observed metformin-associated behavioral improvements involved changes in blood glucose levels. In addition, further tests with metformin administration alone *in vivo,* a potential confound in this study, warrants further investigation.

6. CONCLUSION

We started this study with three objectives: to understand the effect of varying concentrations of CPA *in vivo* and *ex vivo*, to characterise the effects of metformin on hippocampal health after exposure to chronic A1R signalling, and to investigate the effect of metformin on adenosine A1R signalling *in vivo* and *ex vivo*. Our results showed that hypoxia-induced cell death was in part due to adenosine A1R activation. We know this because A1R agonist CPA (100nM) administration produced similar levels of cell death. We found that whether CPA was administered acutely *ex vivo* or chronically *in vivo* it was neurotoxic and augmented cell death. Metformin on the other hand increased fEPSP signalling and conferred neuroprotection both *ex vivo* and *in vivo* during hypoxia and during CPA treatment, respectively. These studies highlighted a potential novel role of metformin during stroke as metformin was able to prevent CPA-induced A1R internalisation upon acute and chronic adenosine stimulation. Therefore, during sustained adenosine release, which normally would be considered neurotoxic, we found metformin was able to prevent both hypoxia-induced cell death, and adenosine-mediated cell death. We attributed this effect to metformin's ability to bind to and antagonize A1Rs, as we found metformin was indeed able to bind to and displace a prototypical A1R antagonist (DPCPX) with an apparent binding affinity of 48nM. Therefore, we believe that metformin acts an A1R antagonist like DPCPX but may also have binding affinities for other adenosine receptors, and that by primarily blocking A1Rs at times where this receptor would be prone to exerting its neurotoxic effects, metformin was able to confer neuroprotection.

7. FUTURE DIRECTIONS

It is imperative that we investigate the potential of metformin to bind to other adenosine receptors like A2A, A2B, and A3 with radioligand experiments. Already in our experiments so far, we have seen metformin exert A2AR antagonist-like effects, including 1) prevention of the A2AR-dependent synaptic potentiation during prolonged normoxic washout following a 20 min hypoxic stimulation, 2) lack of effect on maximal fEPSP inhibition during hypoxia-induced synaptic depression, and 3) relatively low affinity of metformin on A1R binding. This is of note, particularly because A2AR is a receptor of interest in diseases like PD and epilepsy.

Metformin is already clinically approved for T2D, but we believe it could be repurposed for other diseases, and conditions involving adenosine receptors given that we have shown metformin is able to improve motor deficits, reduce post-hypoxic excitability and improve cognition.

Future experiments would also involve characterising signalling molecules of note involved in mediating adenosine receptor pathways (AMPK, PP2A, MAPK, JNK) to better understand how metformin is able to attenuate neurological disease pathogenesis.

It is also our hope to administer metformin following pial vessel disruption (PVD) surgery to assess the therapeutic potential of metformin in a true *in vivo* stroke model.

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